University of Alberta

Extrapituitary Pituitary Hormones: GH and TSH in tissues of the domestic fowl (*Gallus domesticus)*

by

Amy Elizabeth Murphy

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

It is generally thought that pituitary hormones are not involved in early embryonic growth and development, since the pituitary gland does not develop until mid-late gestation. However, pituitary hormones have been localized in many extrapituitary tissues and in some early embryonic tissues. Therefore, extrapituitary "pituitary" hormones may have essential autocrine/paracrine roles in extrapituitary tissues and in the early embryo prior to pituitary differentiation.

Immunoreactive GH- and βTSH-like proteins were detected in numerous extrapituitary tissues of the ED7 (prior to the ontogeny of functional GH-secreting somatotrophs) chick embryo. The distribution of immunoreactive GH was different than that of immunoreactive βTSH. The transcription of both pituitary GH and βTSH is dependent on the transcription factor Pit-1. Immunoreactive Pit-1 like proteins of appropriate size (33kDa) and the Pit-1 transcript were also detected in the ED7 chick embryo. The distribution of Pit-1 immunoreactivity differed from that of GH and βTSH in some tissues. These results suggest GH and βTSH may have autocrine or paracrine roles during embryonic development and their transcription may be Pit-1 dependent in some ED7 chick embryo tissues and Pit-1 independent in others.

GH, GH-receptor and Pit-1 genes were also detected in the adult male chicken reproductive tract and the chicken testicular GH gene was cloned and sequenced revealing a novel chicken testicular GH transcript. These results provide further evidence for a role for growth hormone in reproductive physiology.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
ACTH	adrenocorticotropin
bp	base pairs
cDNA	complmentary dioxyribonucleic acid
cGH	chicken growth hormone
Cys	cysteine
DA	dopamine
DAB	diaminobenzidine
DIG	digoxigenin
ED	embryonic day
FSH	follicle stimulating hormone
Gap DH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
hCS	human chorionic somatomammotropin
hGH-N	human growth hormone-normal
hGH-V	human growth hormone-variant
ICC	immunocytochemistry
IGF	insulin-like growth factor

IHC	immunohistochemistry
IR	immunoreactivity
kDa	kilodalton
LH	luteinizing hormone
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pit-1/GHF-1	pituitary-specific transcription factor-1/ growth hormone factor-1
PRL	prolactin
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulphate
SRIF	somatostatin
T3	L-3,5,3-triiodothyronine
T4	thyroxine
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone

Chapter 1

Literature Review:

Pituitary and Extrapituitary Hormones

I. GENERAL INTRODUCTION: The Expanding Endocrine System

Until recently, the endocrine system (as taught in the medical school curriculum) was thought to comprise a number of scattered glands throughout the body (the hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenal and gonads). By definition, they were characterized by being ductless glands that secreted their products (hormones) into the bloodstream that connected them. Other than this, it was difficult to characterize the endocrine system, as it was evidently comprised of neural and non-neural tissues, discrete glands or scattered cells, and individual, paired or multiple glands that contained one or more secretory cell-type, each of which might secrete one or more hormones. The distinctiveness of the endocrine system became even more problematic when it was realized that the brain, heart, lungs, kidneys, thymus, spleen and liver also had endocrine function and when the skin and cardiovascular system were thought to be the largest endocrine tissues of the body (Peart, 1977; Beh-Harari and Youdim, 1983; Henderson, 1987; Cantin and Genest, 1988; Anggard, 1990; Zouboulis, 2000).

Whilst it is now difficult to describe unique features of the endocrine system, it is equally difficult to define hormones, the classical messages of the endocrine system. Hormones were described as homeostatic regulators that acted at distant target sites, with a latency of response, after transportation through the blood system from their sites of production. This definition does not, however, differentiate "hormones" from "parahormones", like carbon dioxide and glucose (Kolata, 1982; Martin, 1985). Moreover, while a chemical messenger may have endocrine action following its release into the bloodstream, it is now realized that the same chemical may exert local (paracrine) actions on neighboring cells or direct (autocrine) actions on the cell of origin.

It may even be retained within the cell to exert "intracrine" action or be transported through a cellular matrix (juxtacrine) or body cavity (luminocrine) rather than through the bloodstream. These messengers may also act, dogmatically, through signal-transduction system, but any one may act through a variety of plasma membrane, cytoplasmic or nuclear receptors. Some may also act without receptor occupancy, through changes in the conformation of the plasma membrane or via intercellular communications via gap junctions. Many are also now known to act rapidly, with very little latency in the response. Many of these chemical messengers are also now known to be produced in more than one tissue or site within the body.

The ectopic (abnormal) production of hormones in "unusual" tissues is well established, although it was thought to be an epiphenomenon associated with malignancy and other pathologies (Barkan *et al.*, 1986; Morgello *et al.*, 1988; Coleman *et al.*, 2000; Ohta *et al.*, 2000; Indinnimeo *et al.*, 2001). It is, however, now known that many hormones are physiologically expressed in many sites in which they may have systemic or local actions. Thus, while growth hormone (GH), for instance, is generally considered as an endocrine factor produced by the pituitary gland, it is also thought to be a neurocrine, produced within the brain, a cytokine within the immune system and a growth factor in extrapituitary tissues (Render *et al.*, 1995a; Waters *et al.*, 1999; Harvey *et al.*, 2000a; Ramesh *et al.*, 2000). The presence of GH and TSH (thyroid stimulating hormone) (another hormone primarily produced by the pituitary gland) in extrapituitary tissues is the focus of the review and the research described.

II. GROWTH HORMONE (GH)

A. Pituitary GH

1. SOMATOTROPHS

Growth hormone (GH) is primarily produced in pituitary somatotrophs, which are the most abundant adenohypophyseal cell type (Porter, 1997). These cells have geographical and spatial distributions within the pituitary gland that are species-related (Harvey, 1995). In the chicken, most somatotrophs (>90%) are found in the caudal lobe of the adenohypophysis and account for more than 40% of the secretory cell types in the lobe (Hull *et al.*, 2000). The distinct cellular location of these cells therefore promotes paracrine relationships between somatotrophs and folliculostellate cells and other secretory cells types (Schwartz *et al.*, 1998). Corticotrophs are, for instance, closely associated with somatotrophs in the rat pituitary (DeNef, 1986), and adrenocorticotropin (ACTH) and GH reciprocally regulate somatotroph and corticotroph function (Schwartz and Perez, 1994).

Somatotrophs are characteristically large, ovoid shaped cells with large nuclei, although morphological heterogeneity is present in many species, including the domestic fowl (Hull *et al.*, 2000). This morphological heterogeneity may be related to differences in the composition of the intracellular organelles and to the production of heterogeneous GH moieties. This heterogeneity may also reflect the presence of cells that secrete more than one pituitary hormone: prolactin (PRL) – (mammosomatotrophs), thyrotropin (TSH) – (thyrosomatotrophs), ACTH – (corticosomatotrophs) and luteinizing hormone (LH) – (gonadosomatotrophs) having been identified in mammalian pituitary glands (Frawley

and Boockfor, 1991; Childs, 2000; Vidal *et al.*, 2000). The presence of GH-secreting and non-GH-secreting somatotrophs (silent somatotrophs, Frawley and Boockfor, 1991) and of GH depleted and GH replete cells (Hull *et al.*, 2000) also contribute to somatotroph heterogeneity because of differences in GH content and immunocytochemical staining.

2. CHEMISTRY

The most abundant form of GH in the pituitary gland is a single chain 22 kilodalton (kDa) protein of approximately 191 amino acids. The basic GH structure is highly conserved amongst species and is characterized by 4 cysteine residues that form two disulfide bridges (Cys^{53}-Cys^{165} and Cys^{182}-Cys^{189}) and create large and small intramolecular loops, respectively (Charrier and Martal, 1988). These loops are thought to be important for receptor binding and signal transduction, although specific biological activities are thought to reside within discrete polypeptide fragments of the intact molecule (Lewis, 1984; Campbell and Scanes, 1995), suggesting the presence of multiple GH receptor (GHR) moieties.

In addition to 22 kDa GH (monomer GH), numerous GH moieties are also present in the pituitary gland (Aramburo *et al.*, 2000; 2001). These are thought to reflect differences in gene transcription or post-translational modifications of the protein. In chickens, these include charge variants (Aramburo *et al.*, 1989), glycosylated GH (Berghman *et al.*, 1987), phosphorylated GH (Aramburo *et al.*, 1990; 1992), oligomeric forms (dimers, trimers, quatramers and pentamers; Lewis, 1984; Stolar, 1984), and proteolytic cleavage forms (Aramburo *et al.*, 1989; Render *et al.*, 1995b). They are also known to be differentially produced in response to different GH-secretagogues

(Martinez-Coria *et al.*, 2002) and their relative abundance changes during ontogeny (Aramburo *et al.*, 2000).

3. GENE

In humans, the pituitary GH gene (hGH-N) is a member of a family of five highly homologous genes that are encoded within 55 kilobases (kb) on band q22-q24 on the long arm of chromosome 17 (Parks, 1989). In addition to hGH-N these genes include human chorionic somatomammotropin (hCS-A and hCS-B) (also called placental lactogens), a hCS-pseudogene (hCS-L, that is not transcribed) and a placental hGH variant (hGH-V, which is present as hGH-V_1, hGH-V_2, and hGH-V_3 proteins) (MacLeod *et al.*, 1992; Lytras *et al.*, 1994; Misra-Press *et al.*, 1994; Boguszewski *et al.*, 1998).

These genes are differentially expressed in tissue- and time-dependent ways (Nickel *et al.*, 1990; Eberhardt *et al.*, 1996). The hGH-N and hGH-V genes are both expressed in the pituitary gland and placenta, although the hGH-N gene is preferentially expressed in the pituitary while the hGH-V gene is preferentially expressed in the placenta. Placental GH-like genes have also been found in sheep (Lacroix *et al.*, 1996) and rats (Ogilvie *et al.*, 1990). Two GH genes have also been identified in the pituitary glands of some fish (Agellon *et al.*, 1988; Lorens *et al.*, 1993; Law *et al.*, 1996), in which a somatolactin gene (resembling both GH and prolactin) may additionally be expressed (Rand-Weaver *et al.*, 1991; Astola *et al.*, 1996).

In addition to heterogeneous GH genes, the presence of two promotors for the hGH-N gene (Courtois *et al.*, 1992; Giordano *et al.*, 1997; Rodrigues *et al.*, 1998) indicates the existence of transcriptional heterogeneity. Alternate splicing of the hGH-N

pre-mRNA (in which exon 2 of the five exons is spliced to an alternative acceptor site located 45 bases within exon 3) results in the synthesis of a 20 kDa protein lacking 15 internal amino acid residues (32-46, inclusive) (Lewis *et al.*, 1980), indicating the presence of post-translational heterogeneity.

In birds, the pituitary GH gene has been cloned from the chicken (Zhvirblis *et al.*, 1987; Baum *et al.*, 1990; Tanaka *et al.*, 1992), turkey (Foster *et al.*, 1991), and duck (Chen *et al.*, 1988). These have close homology (>90% similarity) with each other and with mammalian GH genes (>60% homology) (Tanaka *et al.*, 1992). A unique transcript, coding for a truncated 16 kDa GH moiety (lacking the amino terminus of monomer GH), has also been identified in the retina of embryonic chicks (Takeuchi *et al.*, 2001). Numerous polymorphisms of the GH gene, as a result of differences in intron sequences (Kuhnlein *et al.*, 1997; Ip *et al.*, 2001), have also been identified in the chicken pituitary. Some of these polymorphisms are strain related and correlate with differences in egg production (Kuhnlein *et al.*, 1997; Ip *et al.*, 2001).

4. ONTOGENY

Embryonic or fetal growth is thought to be a "*growth-without-GH*" syndrome (Geffner, 1996), since much of it occurs prior to pituitary differentiation and the onset of GH secretion.

Although somatotrophs account for >40% of the cells in the caudal lobe of 4-6 week old chickens (Malamed *et al.*, 1988) immunoreactive somatotrophs are generally thought to be absent until mid-development (between embryonic day (ED) 10 or ED 12 of the 21 day incubation period), and even then they are sparse (0.2% of parenchymal

cells), small and poorly developed, with pleiomorphic secretory granules (Porter, 1997; Jozsa *et al.*, 1979). The number, size and content of the secretory granules increases towards hatch, but mature, spherical secretory granules are not present until ED 15. The number of somatotrophs also increases towards hatch, when they account for almost 20% of the pituitary cells (Malamed *et al.*, 1993). An earlier ontogeny of somatotrophs, in ED 7-ED 8 chick embryos, was reported by Gasc and Sar (1981) and Mikami and Takahashi (1987). Thommes *et al.* (1987) even suggested somatotrophs were present in Rathke's pouch in ED4.5 embryos, although others have concluded they are not present until ED 13 (Barabanov, 1991), ED 15 (Wingstrand, 1951), ED 18 (Tixier-Vidal, 1954) or ED 19 (Rahn, 1939). This controversy may reflect differences in methodology, but it is consistent with the ontogeny of somatotrophs in mammalian pituitary glands, which has been reported to occur mid-way through the first trimester of gestation in some studies (Baker and Jaffe, 1975; Asa *et al.*, 1986), but mid-way through the last trimester in other studies (Chatelain *et al.*, 1979; Wilson and Wyatt, 1993). It is, however, clear that the ontogeny of pituitary somatotrophs occurs after the onset of organogenesis, which must therefore occur in the absence of pituitary GH.

The ontogeny of pituitary somatotrophs occurs coincident with the ontogeny of GH synthesis. In the chicken, transcripts of the GH gene are present in the pituitaries of ED 12 embryos and increase in abundance towards hatch (Kansaku *et al.*, 1994; McCann-Levorse *et al.*, 1993). This induction of GH gene transcription is preceded by increased levels of the pituitary-specific transcription factor (Pit-1), which is thought to be essential for pituitary GH gene expression (Harvey *et al.*, 2000b). The abundance of the pituitary GH transcript in ED 18 chick embryos is, however, <20% of that in hatched

chicks and <3% of that in 4-week-old birds. The GH content of the pituitary gland similarly doubles between ED 12 and ED 18, but even then it is <10% of that in newly hatched chickens and <1% of that in 3-week-old birds (McCann-Levorse *et al.*, 1993). The expression of the GH gene is therefore minimal during embryonic development.

The ontogeny of pituitary somatotrophs also precedes the ontogeny of pituitary GH secretion. While immunoreactive somatotrophs have been identified in the pituitaries of ED 10 and ED 12 embryos (Porter, 1997), GH-secreting somatotrophs (detectable by the reverse hemolytic plaque assay) are not thought to differentiate until ED 14 and ED 16 (Porter *et al.*, 1995a,b). Somatotroph responsiveness to GH-releasing stimuli also only occurs during late embryogenesis, since the hypothalamus-pituitary axis does not become a functional unit until 1 week before hatching (Decuypere and Scanes, 1983; Thommes *et al.*, 1983; Porter *et al.*, 1995a). It is, therefore, not surprising that the secretion of GH into peripheral circulation does not occur until after ED 16 (Harvey *et al.*, 1998). Pituitary GH cannot, therefore, exert endocrine actions on tissue growth until late embryogenesis. It is, however, possible that GH produced in extrapituitary tissues prior to pituitary differentiation might be involved in early embryonic development (Harvey *et al.*, 1998).

B. Extrapituitary GH

1. DISTRIBUTION

It is now well established that the expression of the GH gene is not confined to the pituitary gland and occurs, in lesser amounts, in extrapituitary tissues (reviewed by Harvey and Hull, 1997; Hull and Harvey, 1998). For instance, in adult chickens, GH and

GH mRNA have been detected in the brain (Render *et al.*, 1995a), in which GH-immunoreactivity is widely distributed (Ramesh *et al.*, 2000). GH and GH mRNA are also present in the thymus, bursa and spleen of adult chickens (Render *et al.*, 1995b). Similarly, in mammals, GH and GH mRNA are present in the nervous system – Harvey *et al.*, 1993; Yoshizato *et al.*, 1998; immune system – Maggiano *et al.*, 1994; Recher *et al.*, 2001; integumentary system – Palmetshofer *et al.*, 1995; Slominski *et al.*, 2000; respiratory system – Allen *et al.*, 2000; alimentary system – Tresguerres *et al.*, 1999; Kyle *et al.*, 1981; cardiovascular system – Costa *et al.*, 1993; Wu *et al.*, 1996; Recher *et al.*, 2001; reproductive system – Hu *et al.*, 1999; Schwarzler *et al.*, 1997; Untergasser *et al.*, 1997; mammary glands – Mol *et al.*, 1995a;b; and teeth – Zhang *et al.*, 1997).

2. CHEMISTRY

Since extrapituitary GH has largely been detected immunologically, it must share epitopes with pituitary GH. It is also associated with proteins comparable in size to monomer GH in the pituitary gland (Render *et al.*, 1995a,b) and with numerous size variants (Render *et al.*, 1995a,b; Aramburo *et al.*, 2000; Luna *et al.*, 2002). However, the GH-immunoreactivity in the rat brain is more akin to human GH than to rat pituitary GH (Hojvat *et al.*, 1982), and this may indicate tissue-specific differences in GH structure (Harvey *et al.*, 1993).

3. GENE

The GH gene in the human (Martial *et al.*, 1979), rat (Seeburg *et al.*, 1977) and chicken (Tanaka *et al.*, 1992) has been cloned and sequenced. In all cases it consists of 5

exons and 4 introns. The GH cDNA in chicken (Render *et al.*, 1995b), rat (Weigent and Blalock, 1991) and human (Melen *et al.*, 1997) immune tissues is identical to that in the corresponding pituitary glands. The GH gene in the human placenta (Boguszewski *et al.*, 1998), testis (Berger *et al.*, 1999) and ovary (Schwarzle *et al.*, 1997; Untergasser *et al.*, 1997) does, however, include the placental variant (hGH-V) that codes for hGH-V_1, hGH-V_2 and hGH-V_3. A novel, truncated GH transcript has also been identified in the eyes of chick embryos (Takeuchi *et al.*, 2001). These authors cloned a novel GH cDNA (s-cGH) that consisted of two exons; the first corresponds to a part of intron 3 and exon 4 of the pituitary GH gene, the second exon corresponds to exon 5 of pituitary GH mRNA. The portion coded by this cDNA had a deduced molecular size of 16.5 kDa and 140 amino acid residues, lacking the signal peptide and the N-terminal 71 amino acids of 22 kDa pituitary GH. Its N-terminus would have a unique sequence of 20 amino acids, whereas the C-terminal 120 amino acids (positions 97-216 of the GH prohormone) would be identical to 22 kDa pituitary GH and contain helixes 3 and 4 that bind to the GH receptor (GHR). The conserved four cysteine residues in the C-terminus would permit the formation of two intramolecular disulfide bridges and a secondary (coil) structure as in the corresponding region of 22 kDa pituitary GH. These structural similarities of s-cGH with 22 kDa pituitary GH suggests it could bind to, and activate, tissue GHRs. Interestingly, as the leucine residues in the third helical domain of the s-cGH are arranged every 7 amino acids, it also has the characteristic structure of a leucine-zipper transcription factor and it may thus participate in gene regulation. However, as the N-terminus is hydrophilic and unlikely to serve as a secretory signal peptide, s-cGH

probably acts as an intracrine within the cells expressing this GH isoform. The identity of the cells expressing this isoform is, however, unknown.

4. ONTOGENY

Prior to the appearance of GH-immunoreactivity in pituitary somatotrophs (at approximately ED 12; Porter, 1997) and in Rathke's pouch of chick embryos (at ED 4.5; Thommes *et al.*, 1987; at ED 7; Harvey *et al.*, 2000a) GH-immunoreactivity is present in the endoderm and ectoderm layers of ED 1 and ED 2 embryos (Wang, 1989). Harvey *et al.* (2000a) similarly found that GH immunoreactivity was almost ubiquitously present in peripheral tissues of ED 3 chick embryos and was widespread in ectodermal, mesodermal and endodermal derivitives at ED 5, although not present in every cell of each tissue. Immunoreactive GH was particularly abundant in the neural tube, notochord, limb bud, somites, heart, stomach, liver, kidney, Wolffian duct and the amnion. By ED 8, GH-immunoreactivity was still widespread and now present in limb bud chondrocytes and osteocytes. The presence of GH-immunoreactivity in the Mullerian ducts of male and female chick embryos between ED 5 and ED 12 was also reported by Wang (1989). This immunoreactivity persists in most tissues until at least mid-late embryogenesis (Harvey *et al.*, 1998), and persists in neural (Render *et al.*, 1995a; Ramesh *et al.*, 2000; Harvey *et al.*, 2000a) and immune (Render *et al.*, 1995b) tissues neonatally and through sexual maturity. The GH-immunoreactivity in some tissues (eg. the heart, liver, muscle and mesonephros) is, however, lost by ED 12 – ED 18 of embryonic development (Harvey *et al.*, 1998; 2000a), suggesting tissue-specific regulation of the extrapituitary GH gene.

In the embryonic chick, GH-immunoreactivity was identified by its cross-reactivity with four antisera raised against chicken GH and by a lack of staining following the preabsorption of the antisera with recombinant chicken GH. Western blotting also showed that this immunoreactivity was associated with proteins identical in size to monomer (26.5 kDa) and dimer (53 kDa) GH in the pituitary glands of adult chickens (Harvey *et al.*, 1998) and was present in the trunks of ED 7 embryos (Harvey *et al.*, 1998).

The detection of GH-immunoreactivity in early chick embryos occurred prior to the differentiation of pituitary somatotrophs. It also occurred before the appearance of GH in peripheral plasma (on ED 17; Harvey *et al.*, 1979; Harvey *et al.*, 1998) and, indeed, prior to the maturation of the cardiovascular system (before ED 4). This GH immunoreactivity is therefore likely to reflect an extrapituitary expression of a GH gene. This possibility is supported by the detection, by RT-PCR, of GH mRNA in the bodies of ED 2 – ED 5 chick embryos (Harvey *et al.*, 2000b; 2001a) and in the headless bodies of ED 7 embryos (Harvey *et al.*, 1998). As Pit-1 mRNA was also detected in the same tissues (Harvey *et al.*, 2000b), this early expression of the GH gene may be dependent on an earlier or contemporaneous expression of the Pit-1 gene, since GH gene expression is thought to be Pit-1 dependent.

In addition to embryonic chicks, GH and GH mRNA have been detected in preimplantation mouse embryos (Pantaleon *et al.*, 1997). Yang *et al.* (1999) similarly found GH-immunoreactivity and GH mRNA in trout embryos during gastrulation and organogenesis, although they did not determine its tissue distribution during ontogeny. Izadyar *et al.* (2000) have also found GH and GH mRNA in bovine embryos prior to

blastocyst formation and blastocyst hatching. Hojvat *et al.* (1982a) had earlier found that the appearance of GH-immunoreactivity in the brains of fetal rats occurs before its ontogenic appearance in the pituitary gland. A widespread expression of the GH gene in peripheral tissues of the rat fetus (eg. in gut, liver, muscle and the cardiovascular system) has also been observed (Costa *et al.*, 1993; Recher *et al.*, 2001), even though GH gene expression in these tissues is not seen neonatally. GH gene expression therefore appears to be widespread during early development and before the ontogeny of pituitary GH expression. It is therefore likely that GH acts as a local growth factor during early development rather than as a pituitary endocrine.

5. PARACRINE/AUTOCRINE ROLES?

In addition to GH, GHRs have been identified in many extrapituitary sites of GH production (Harvey and Hull, 1997; Hull and Harvey, 1998). It is therefore likely that GH may have local autocrine/paracrine actions in those sites. For instance, the presence of GH and GHR in the skin (Lobie *et al.*, 1990a; Garcia-Aragon *et al.*, 1992; Costa *et al.*, 1993; Palmetshofer *et al.*, 1995; Slomiski *et al.*, 2000) suggests the presence of an autocrine/paracrine system of GH action in the integument. The presence of GH and GHR in the nervous system (Harvey *et al.*, 2001a), respiratory system (Garcia-Aragon *et al.*, 1992; Costa *et al.*, 1993; Batchelor *et al.*, 1998; Allen *et al.*, 2000), digestive system (Kyle *et al.*, 1981; Lobie *et al.*, 1990b; Garcia-Aragon *et al.*, 1992; Costa *et al.*, 1993; Mori and Devlin, 1999), immune system (Kyle *et al.*, 1981; Lobie *et al.*, 1990a; Costa *et al.*, 1993; Wu *et al.*, 1996), and the musculoskeletal system (Kyle *et al.*, 1981; Garcia-Aragon *et al.*, 1992; Werther *et al.*, 1993; Zhang *et al.*, 1997; Harvey *et al.*, 1998; 2000a)

similarly suggests autocrine/paracrine roles for GH in these tissues. This possibility is supported by the presence of inducible GH-response genes (eg. GH-response gene-1 and insulin-like growth factor-1) in many of these tissues (Harvey *et al.*, 2001a,b). Moreover, in many tissues, local actions of GH have been demonstrated that are blocked by GH-immunoneutralization. For instance, the blockade of endogenous GH in fetal rats prevents the differentiation of the Wolffian duct (Nguyen *et al.*, 1996). GH-immunoneutralization similarly blocks the GH-induced glucose transport in preimplantation mouse embryos (Pantaleon *et al.*, 1997), the hatching of murine (Drakakis *et al.*, 1995; Fukaya *et al.*, 1998) and bovine (Izadyar *et al.*, 2000) blastocysts, haemopoietic function in human lymphocytes (Sabharwal and Varma, 1996) and mammary development in dogs (Mol *et al.*, 1996). Antisense oligonucleotides for the GH gene similarly block the actions of endogenous GH in the rat immune system (Weigent *et al.*, 1991). The local production of GH in cultured *BRL* cells is similarly unable to prevent apoptosis following transfection with a mutant GHR gene (Liu *et al.*, 1997).

III. THYROID STIMULATING HORMONE (TSH)

A. Pituitary TSH

1. THYROTROPHS

Pituitary TSH is synthesized in, and secreted from, basophilic cells called thyrotrophs (Nathanielsz, 1976). Thyrotrophs are stellate shaped and contain TSH secretory granules up to 200 nm in diameter (Harrisson *et al.*, 1982). The Golgi bodies and the endoplasmic reticulum are well developed and extensive, suggesting high TSH

synthetic activity. Thyrotrophs occur in low abundance in the pituitary gland, accounting for only 1-2% of cells in the rat adenohypophysis (Manger, 1989) and about 2% of the cells in the late chick embryo (Porter *et al.*, 2000). In Aves, a restricted distribution of thyrotrophs has been seen in the cephalic (or rostral) lobe of the anterior pituitary gland, although other investigators have localized TSH in both lobes (Table 1.1). Thyrotrophs are not a fixed population and can increase by both hyperplasia and hypertrophy in response to increased stimulation (Torres *et al.*, 1995).

2. CHEMISTRY

Thyroid stimulating hormone (TSH), or thyrotropin, is the primary regulator of the thyroid gland. TSH stimulates the release of the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), (Radke and Chiasson, 1977). TSH, like luteinizing hormone (LH), follicle stimulating hormone (FSH) and chorionic gonadotropin (CG) is a heterodimeric glycoprotein consisting of noncovalently bound α and β subunits (Holdstock and Burrin, 1994). The α subunit is common to all glycoprotein hormones and conveys species specificity, while the β subunit is specific to each hormone and determines hormonal activity (Pierce and Parsons, 1981; Querat, 1994). The α and β subunits are synthesized as separate moieties from different genes (Hsieh *et al.*, 2000). The TSH holoprotein molecular weight is estimated at 25 – 33 kDa for mammals (Pierce, 1971; Jacobson *et al.*, 1977) and 28.3 – 38 kDa for chickens (Prada *et al.*, 2000; Porter *et al.*, 2000). Subunit molecular weights are estimated at 18 – 22 kDa in mammals (Pierce and Parsons, 1981) and 17 – 18 kDa in chickens (Krishnan *et al.*, 1994; Porter *et al.*, 2000). Different isoforms of TSH exist and differ in biological activity. However, the

differences in the isoforms appear to be in oligosaccharide structure and not in the peptide backbone (Beck-Reccoz and Persani, 1994; Szkudlinski *et al.*, 1995).

3. GENE

The chicken βTSH gene has been cloned and the cDNA sequence determined (Gregory and Porter, 1997: Kato *et al.*, 1998). It has 93-94% homology with TSH from other avian species (e.g. the duck, Hsieh *et al.*, 2000; quail, Kato *et al.*, 1997), 67-69% homology with mammalian TSH (e.g. human TSH, Guidon *et al.*, 1988; bovine TSH, Maurer *et al.*, 1984; rat TSH, Croyle and Maurer, 1984), 61% homology with frog TSH (Buckbinder and Brown, 1993); and lesser homology (44-52%) with TSH from teleosts (e.g. trout, Ito *et al.*, 1993; salmon, Martin *et al.*, 1999; eel, Salmon *et al.*, 1993). There are three areas within the βTSH cDNA sequences that are highly conserved among vertebrates, and Area I is thought to have an important function concerning the mechanism of βTSH action (Gregory and Porter, 1997).

4. CONTROL OF PITUITARY TSH

TSH synthesis and secretion are controlled by hypothalamic and thyroid hormones. Thyrotropin releasing hormone (TRH) increases TSH secretion from the pituitary of chickens *in vitro* (Scanes, 1974; Breneman and Rathcamp, 1973) and increases βTSH mRNA in ducks (Hsieh *et al.*, 2000). T_4 and T_3 downregulate βTSH mRNA formation in chickens and ducks via negative feedback (Gregory and Porter, 1997; Hsieh *et al.*, 2000). Somatostatin (SRIF) and dopamine (DA) also have inhibitory

effects on TSH synthesis and secretion (Chin *et al.*, 1993), which is also suppressed by gonadal steroids (Tixier-Vidal and Assenmacher, 1961, Tixier-Vidal *et al.*, 1972).

5. ONTOGENY

Thyrotrophs appear ontogenically prior to somatotroph, lactotroph and gonadotroph differentiation in mammals (Burrows *et al.*, 1999) and birds (Porter, 1997). A large number of transcription factors are located in Rathke's pouch and required for the formation of thyrotrophs, as well as other hormone secreting cells, and include *Hesx1*, *Pitx1*, *Msx1*, *Lbd1,3*, *Pit1*, *Lhx2*, *Lhx3*, *Pitx2*, *Gata2* and *Egr1* (reviewed by Burrows *et al.*, 1999). Thyrotroph embryonic factor (TEF) also activates the TSHβ gene promotor (Drolet *et al.*, 1991).

Embryonic chick thyroids cultivated *in vitro* first respond to TSH stimulation on ED 6 (Tixier-Vidal, 1959) or ED 6.5 (Thommes and Hylka, 1978), and embryonic chick pituitary glands are responsive to TRH stimulation (as indicated by increased circulating T_4 levels) by ED 6.5 (Thommes and Hylka, 1978). However, normal thyroid activity does not become pituitary dependant until between ED 10 and 12, when TSH becomes abundant in the pituitary gland (Thommes and Hylka, 1978). Pituitary βTSH mRNA is not present until ED 11 and increases toward hatch with a high peak on ED 19 (Gregory *et al.*, 1998). In contrast, Geris *et al.* (1998) found that plasma TSH indices dropped toward hatch. Thommes *et al.* (1983) found immunoreactive βTSH in Rathke's pouch at ED 6.5, but it remained in low abundance until ED 11.5, when both lobes of the anterior pituitary gland were distinct.

6. ACTIONS

The major function of pituitary TSH in the embryo is thought to be the regulation of thyroid function. Pituitary TSH begins to regulate thyroid activity in the chick embryo between ED 10.0 and ED 12.0 (Thommes and Hylka, 1977). Circulating T_4 levels fail to increase after ED 10 – 11.5 in hypophysectomized chicks (Thommes *et al.*, 1977), indicating pituitary control of T_4 release at this stage. Iqbal *et al.* (1987) found that chronic TSH exposure in ED 15 chicks stimulates increased thyroid peroxidase activity, and Hamori *et al.* (1959) demonstrated increased thyroid iodine uptake after ED 11 after surgical decapitation reduced thyroid ^{131}I uptake rates after ED 11. Experiments by Kuhn *et al.* (1988) showed that TSH stimulation in ED 17 and 18 chicks increased T_3, T_4 and reverse (r) T_3 levels, but did not increase hepatic 5'-monodeiodination (5'-D) activity.

TSH is known to have other actions in the embryo unrelated to thyroid stimulating activities. TSH is the dominating lipolytic hormone *in vitro* during the neonatal period (Marcus *et al.*, 1988) and it regulates lipolysis in neonatal human adipocytes to help with the metabolic adaptation after birth (Janson *et al.*, 1998). Haraguchi *et al.* (1999) have also found that TSH induces proliferation and inhibits differentiation of cultured rat preadipocytes, and Bell *et al.* (2000) speculate a role for TSH in adipose development. In addition, TSH is known to have roles in embryonic gonadal tissues. TSH stimulates growth and differentiation of the gonad (Csaba *et al.*, 1980; Shanin *et al.*, 1982a,b), migration of germ cells to the gonads (Shanin *et al.*, 1982b) and mitosis of the oocyte (Csaba *et al.*, 1980).

B. Extra-Pituitary TSH

TSH and TSHRs are expressed in a variety of tissues in different species (Table1.2 and 1.3). Extra-pituitary TSH may be produced locally within these tissues and act as an autocrine or paracrine in these sites.

1. TSH: A NEURAL AUTOCRINE/PARACRINE?

The brain is thought to be an extra-pituitary site of TSH synthesis since brain TSH is present prior to pituitary TSH in the rat (Hojvat *et al.*, 1982a,b), so brain TSH is unlikely to be from peripheral blood. This possibility is also supported by the pituitary independent changes in brain TSH concentration that occur diurnally in rats (Ottenweller and Hedge, 1982), during growth (Emanuele *et al.*, 1985), and with endocrine manipulations (Hojvat *et al.*, 1985; De Vito *et al.*, 1985).

Neural TSH may act in an autocrine or paracrine manner since TSH receptor (TSHR)-like protein and TSHR mRNA are also found in the brains of mammals (Labudova *et al.*, 1999; Crisanti *et al.*, 2001; Bockmann *et al.*, 1997a,b) and fish (Kumar *et al.*, 2000). Indeed, TSH has been shown to have actions in neural tissues. In astroglial cells TSH stimulates the release of arachidonic acid and the activity of type II 5'-iodothyronine-deiodinase (Saunier *et al.*, 1993) and stimulates mitogen-activated protein kinase isozymes (Tournier *et al.*, 1995). TSH may also regulate TSHR expression in the brain (Crisanti *et al.*, 2000). It has, furthermore, been speculated that TSH may act as a neuromodulator in the human retina, and to regulate retinal blood flow (Fernandez-Trujillo *et al.*, 1996). It may also act as a modulator of synaptic transmission and a regulator of trophic function in the chicken retina (Prada *et al.*, 2000).

2. TSH: AN IMMUNE AUTOCRINE/PARACRINE?

Immune tissues have been found to produce TSH. Human lymphocytes produce a TSH-like peptide (Smith *et al.*, 1983) and express a βTSH gene (Peele *et al.*, 1993). Harbour *et al.* (1989) have demonstrated TSH mRNA in, and *in vitro* TSH secretion from, T cell lines. In addition, Bodey *et al.* (2000) have shown TSH secretion from human thymic reticulo-epithelial cells. Extra-pituitary immune TSH likely acts in an autocrine or paracrine manner, since TSH receptor mRNA and protein have been found extensively in immune cells and TSH is known to have effects in immune function.

TSHRs have also been located in the immune systems of mammals (Table 1.3) suggesting that immune TSH may act in an autocrine or paracrine manner. TSH has many stimulatory effects on the immune system and it has been speculated that the primary source of immune TSH is not the pituitary gland, but the immune system itself (Bagriacik and Klein, 2000). TSH has been shown to increase the cytotoxic activity of natural killer (NK) cells and to act as a costimulatory factor for T-cell proliferation (Provinciali *et al.*, 1992). It also enhances phagocytic activity and cytokine responses of TSH stimulated murine dendritic cells (Bagriacik and Klein, 2000), as well as increasing cytokine responses of hematopoietic cells (Whetsell *et al.*, 1999; Delgado *et al.*, 1998). TSH can alter the immunological composition of lymphoid cells in the intestinal epithelium (Wang *et al.*, 1997). In addition, TSH modulates human lymphocyte activation, as demonstrated by blocking TRH stimulation with administration of an anti-ratTSH antibody (Raiden *et al.*, 1995).

3. TSH: A PITUITARY AUTOCRINE/PARACRINE?

The pituitary gland is the major site of TSH synthesis and secretion. Recently TSHRs have been localized in the pituitary gland suggesting autocrine/paracrine roles for TSH in pituitary function. Prummel *et al.* (2000) and Theodoropoulou *et al.* (2000) found TSHR mRNA and protein in folliculostellate cells and thyrotrophs of the human pituitary. Together, these results indicate the presence of a short-loop feedback mechanism through which pituitary thyrotrophs autoregulate their synthesis and secretion of TSH (Theodoropoulou *et al.*, 2000), and paracrine actions of TSH on the function of folliculostellate cells (Prummel *et al.*, 2000). The concept of pituitary hormone autoregulation is supported by other studies identifying prolactin (Jin *et al.*, 1997) and GH (Mertani *et al.*, 1995) receptors in the pituitary gland.

4. EMBRYONIC EXTRAPITUITARY TSH

Extrapituitary TSH in the embryo or fetus has, thus far, only been localized in the extahypothalamic rat brain (Hojvat *et al.*, 1982a). Brain TSH is not considered to be of pituitary origin since circulating radiolabeled TSH does not cross the blood-brain barrier in the fetus and radiolabeled TSH in the mother does not cross into fetal circulation (Hojvat *et al.*, 1982a). βTSH protein was detected in the fetal rat brain at ten days of fetal life, five days prior to its detection in the anterior pituitary at fifteen days of fetal life, and persists throughout development (fetal, neonatal, prepubescent and adult) of the rat brain (Hojvat *et al.*, 1982a). Roles for brain TSH in the fetal rat have yet to be determined; however, actions for neural TSH in adults have been suggested (Theodoropoulou *et al.*, 2000).

IV. PITUITARY-SPECIFIC TRANSCRIPTION FACTOR-1 (PIT-1)

A. General Introduction

Pituitary-specific transcription factor-1 (Pit-1), or growth hormone factor-1 (GHF-1), is a member of the POU-homeodomain family of proteins. It binds to and transactivates promoters of growth hormone, prolactin and β-thyroid stimulating hormone genes (Bodner *et al.*, 1988). Pit-1 also autoregulates its own expression, while the initial expression of Pit-1 is induced by the Prophet of Pit-1 (PROP-1) gene (Sornson *et al.*, 1996). In mammals, Pit-1 is a 33 kDa, 291 amino acid protein that binds to two adjacent sites in the GH, PRL and βTSH promoter regions and binding to both sites is necessary for transcription (Harvey *et al.*, 2000b).

The Pit-1 gene has been cloned and sequenced in mammals (bovine, Bodner *et al.*, 1988; rat, Bodner *et al.*, 1988, Ingraham *et al.*, 1988; mouse, Li *et al.*, 1990; swine, Tuggle *et al.*, 1993; human, Tatsumi *et al.*, 1992) and several transcripts have been identified. These include, in addition to the major Pit-1, moiety Pit-1α, the variants Pit-1β, Pit-1T, Pit-1γ, Pit-1Δ3 and Pit-1Δ4, which arise by alternative transcription, initiation and alternative splicing (Theill *et al.*, 1992; Konzak and Moore, 1992; Haugen *et al.*, 1993), and act differently on the promoters of target genes. In Aves, Pit-1 has been cloned and sequenced from chicken (Tanaka *et al.*, 1999; Van As *et al.*, 2000) and turkey (Wong *et al.*, 1992; Kurima *et al.*, 1998) pituitary glands. Three different transcripts have been identified in the turkey: tPit-1, tPit-1β and tPit-1W (Kurima *et al.*, 1998) and in the broiler chicken: ggPit-1, ggPit-1β and ggPit-1W (Van As *et al.*, 2000), although Tanaka *et al.* (1999) only identified two Pit-1 transcripts (cPit-1α, homologous to ggPit-

1, and cPit-1γ, homologous to ggPit-1W) in a layer strain of chicken. The three chicken Pit-1 transcripts have a high degree of homology with the three turkey Pit-1 transcripts (Van As *et al.*, 2000). The mammalian Pit-1 mRNA consists of six exons and five introns, while turkey Pit-1 mRNA consists of seven exons and six introns (Kurima *et al.*, 1998).

Pit-1 expression is present in the pituitary gland of mammals prior to GH, PRL and βTSH expression (Burrows *et al.*, 2000), and is in fact necessary for the ontogeny of somatotrophs, lactotrophs and thyrotrophs (Camper *et al.*, 1990; Li *et al.*, 1900; Castrillo *et al.*, 1991). This is demonstrated in Pit-1 deficient dwarf Snell and Jackson mice, which have dysfunctional mutations of the Pit-1 gene (Cohen *et al.*, 1996), and Ames mice, which have a mutation in the PROP-1 gene that results in Pit-1 deficiencies (Anderson and Rosenfeld, 1994). Both strains of mice are deficient in somatotrophs, lactotrophs and thyrotrophs (Chen *et al.*, 1990). In chickens, Pit-1 mRNA has been localized in the pituitary as early as embryonic day (ED) 5, which was thought to precede the gene expression and secretion of GH, PRL and βTSH (Van As *et al.*, 2000).

B. Pituitary Specificity?

Pit-1 mRNA is present in all five anterior pituitary hormone secreting cell types of the human, porcine and rat pituitary (Malagon *et al.*, 1996), although the Pit-1 protein was thought to be localized only in somatotrophs, lactotrophs and thyrotrophs (Simmons *et al.*, 1990; Asa *et al.*, 1993; Puy and Asa, 1996), indicating cell specific regulation at a transcriptional level. However, other studies have found Pit-1 protein co-localized in rat gonadotrophs (Vidal *et al.*, 1998) and in all five cell types of the porcine anterior pituitary

gland (Malagon *et al.*, 1996). Moreover, Lin *et al.* (1994) have identified a Pit-1 independent line of pituitary thyrotrophs that are present in the fetal rat but disappear in the adult. This suggests that some fetal pituitary cells expressing GH, PRL and βTSH may do so independently of Pit-1.

Pit-1 was thought to be specific to the pituitary because early studies indicated that it was absent from intestine, liver, lung, spleen, thyroid, thymus, pancreas, kidney, muscle, heart, testis, prostate, uterus, brain, and neural retina, as well as absent from fibroblasts, hepatocytes, and placental cells (Bodner and Karin, 1987; Ingraham *et al.*, 1988; Simmons *et al.*, 1990; Lew *et al.*, 1993). However, more recent studies have located Pit-1 protein and mRNA in a variety of extrapituitary sites (Table 1.4). Pit-1 is present in the developing nervous system of rats in the brain and neural retina, but is thought to be absent in the adult rat brain (He *et al.*, 1989), although Torner *et al.* (1999) demonstrated the presence of Pit-1 in the brain of female rats at estrous. Pit-1 has also been localized in immune tissues. Pit-1 mRNA has been shown, by RT-PCR and Southern blotting, to be present in human and rat bone marrow, and in human, rat and chicken spleen and thymus (Table 1.4). Similarly, a Pit-1 mRNA isoform has been found in fetal bovine thymocytes and splenocytes (Chen *et al.*, 1997), and Kooijman *et al.* (1997b) have found a Pit-1 splice variant in circulating human neutrophils. Since the transcription of pituitary GH, PRL and βTSH is Pit-1 dependent, extrapituitary expression of these hormones may be dependent on Pit-1 as well. The expression of GH, PRL and βTSH in extrapituitary tissues may, however, be Pit-1 independent.

This possibility is supported by several studies. GH gene expression occurs in the bone marrow of the Pit-1 deficient Snell dwarf mouse (Blalock and Weigent, 1994;

Kooijman *et al.*, 1997b). Also, GH production in the canine mammary gland (Lantinga-van Leeuwen *et al.*, 1999) and in the pars tuberalis (Bockmann *et al.*, 1997a; Sakai *et al.*, 1999b) occurs in the absence of Pit-1. Moreover, although Pit-1 is present in the placenta, Pit-1 has no effect on placental hGH-V expression (Schanke *et al.*, 1997) and is not required for hGH-V expression in peripheral mononuclear blood cells, since this expression occurs in Pit-1 deficient individuals that are unable to express the hGH-N gene (Melen *et al.*, 1997). Similarly, prolactin gene expression occurs in extrapituitary tissues that do not express Pit-1. PRL is expressed in uterine sarcoma cells in the absence of Pit-1 (Gellersen *et al.*, 1995), as well as in kidney and decidual cells (Sun *et al.*, 1994). The expression of prolactin in lymphocytes and endometrial stroma (Gellersen *et al.*, 1995) and the rat brain (Emanuele *et al.*, 1992) is also Pit-1 independent.

Pit-1 may control the expression of some extrapituitary GH, PRL and βTSH since it has been co-localized with GH and PRL in some tissues. For example, Chen *et al.* (1997) and Kooijman *et al.* (1997b) co-localized GH with Pit-1 in bovine lymphoid cells and human neutophils, respectively, while Sakai *et al.* (1999a) found PRL and Pit-1 in murine kidney cells. However, Pit-1 expression in extrapituitary sites might be unrelated to GH, PRL and βTSH expression since Pit-1 is known to have other roles which include regulating the activity of the renin gene (Gilbert *et al.*, 1994; Catanzaro *et al.*, 1994; Germain *et al.*, 1996) and the genes for the somatostatin (Baumeister and Meyerhof, 1998) and GHRH (Lin *et al.*, 1992; Iguchi *et al.*, 1999; Miller *et al.*, 1999; Gaylinn, 1999) receptors.

V. SUMMARY

The growth and development of the vertebrate embryo is a complex process involving many different spatially and temporally produced growth factors. Early embryonic growth is considered to be independent of pituitary hormones since the pituitary gland does not produce and secrete hormones until mid-late gestation in mammals (Wilson and Wyatt, 1993) and until mid-incubation in birds (Thommes and Hylka, 1978; Porter *et al.*, 1995a,b; Gregory *et al.*, 1998). Therefore, early embryonic development must be under the control of locally produced factors exerting their effects in autocrine or paracrine manners.

The presence of "pituitary" hormones in extrapituitary tissues is largely based on protein immunoreactivity. This does not, however, provide much information on their structural characteristics nor on the genes that express these proteins. It could also result from artefactual cross-reactivity in the immunological techniques used. Further characterization of extra-pituitary phenomena is therefore warranted and the focus of the research described in this thesis.

Table 1.1 betaTSH in Avian Anterior Pituitary

Cephalic Lobe	Species	Reference
	chicken	Porter *et al.*, 2000
		Bergman *et al.*, 1993
	drake	Sharpe *et al.*, 1979
	duck	Marchand and Bugnon, 1972, 1973
	Japanese Quail	Mikami *et al.*, 1975

Both Lobes	Species	Reference
	Chinese quail	Harrisson *et al.*, 1982
	duck	Hassan, 1975
		Tixier-Vidal *et al.*, 1961
	goose	Hassan, 1975
	chicken	Radke and Chiasson, 1974
	pigeon	Matsuo *et al.*, 1969
	White-crowned sparrow	Matsuo *et al.*, 1969
	Orange weaver	Gourdji, 1965

Table 1.2 betaTSH in Extrapituitary Tissues

Tissue	Species	mRNA	Protein	Reference
Brain	Monkey		x	Hojvat *et al.*, 1982b
	Rat		x	Hojvat *et al.*, 1982a
			x	Ottenweller and Hedge, 1982
			x	De Vito *et al.*, 1985
			x	De Vito *et al.*, 1989
Retina	Human		x	Fernandez-Trujillo *et al.*, 1996
	Chicken		x	Prada *et al.*, 2000
Immune	Human		x	Smith *et al.*, 1983
		x	x	Harbour *et al.*, 1989
		x		Peele *et al.*, 1993
			x	Bodey *et al.*, 2000
Gut	Rat		x	Wang *et al.*, 1997
CSF	Human		x	Schaub *et al.*, 1977
Amniotic Fluid	Rat		x	Tang *et al.*, 1984
Placenta	Human		x	Harada and Hershman, 1978

Table 1.3 betaTSH receptor in Extrapituitary Tissues

Tissue	Species	mRNA	Protein	Reference
Brain	Human		x	Lubudova *et al.*, 1999
		x		Crisanti *et al.*, 2001
	Sheep	x		Bockmann *et al.*, 1997b
	Rat	x		Crisanti *et al.*, 2001
	Bony Fish	x		Kumar *et al.*, 2000
Immune	Human		x	Coutelier *et al.*, 1990
		x		Francis *et al.*, 1991
		x	x	Dutton *et al.*, 1997
		x		Spitzweg *et al.*, 1999
	Rat	x		Murakami *et al.*, 2001
	Mouse		x	Bagriacik and Klein, 2000
Heart	Human	x		Drvota *et al.*, 1995
	Pig	x		Sellitti *et al.*, 1997
	Mouse	x		Drvota *et al.*, 1995
	Bony Fish	x		Kumar *et al.*, 2000
Muscle	Human		x	Hiromatsu *et al.*, 1996
		x		Busuttil and Frauman, 2002
	Bony Fish	x		Kumar *et al.*, 2000
Fat	Human	x		Hiromatsu *et al.*, 1996
		x		Crisp *et al.*, 1997
		x	x	Bell *et al.*, 2000
	Rat	x		Endo *et al.*, 1993, 1995
	Guinea Pig		x	Gennick *et al.*, 1986
		x		Roselli-Rehfus *et al.*, 1992
eproducti	Chicken		x	Csaba *et al.*, 1980
			x	Shanin *et al.*, 1982a,b
			x	Woods *et al.*, 1991
	Bony Fish	x		Kumar *et al.*, 2000
Kidney	Human	x	x	Dutton *et al.*, 1997
		x		Sellitti *et al.*, 2000
	Mouse	x		Sellitti *et al.*, 2000

Table 1.3 (cont)

Tissue	Species	mRNA	Protein	Reference
Pituitary	Human	x	x	Prummel *et al.*, 2000
		x	x	Theodoropoulou *et al.*, 2000
Adrenal	Human	x	x	Dutton *et al.*, 1997
Skin	Human		x	Daumerie *et al.*, 2002

Table 1.4 Pit-1 mRNA/protein in Extrapituitary Tissues

Tissue	Species	Reference
Brain	Rat	He *et al.*, 1989
	Mouse	Dolle *et al.*, 1990
	Chicken	Yoshizato *et al.*, 1998
Placenta	Human	Bamberger *et al.*, 1995
	Monkey	Schanke *et al.*, 1997
	Rat	Bamberger *et al.*, 1995
		Lee *et al.*, 1996
Spleen	Human	Delhase *et al.*, 1993
	Bovine	Chen *et al.*, 1997
	Rat	Delhase *et al.*, 1993
	Chicken	Harvey *et al.*, 2000b
Bone Marrow	Human	Delhase *et al.*, 1993
	Rat	Delhase *et al.*, 1993
Thymus	Human	Delhase *et al.*, 1993
	Bovine	Chen *et al.*, 1997
	Rat	Delhase *et al.*, 1993
	Chicken	Harvey *et al.*, 2000b
Lymphocytes	Human	Delhase *et al.*, 1993
Neurophils	Human	Kooijman *et al.*, 1997b
Kidney	Murine	Sakai *et al.*, 1999a

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Chapter 2

Extrapituitary βTSH and GH in Early Chick Embryos

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I. INTRODUCTION

The ontogenic appearance of pituitary somatotrophs occurs during mid-late gestation in most mammalian fetuses and towards the end of the first trimester in human pregnancy (Harvey *et al.*, 1997). Early embryonic and fetal growth is therefore independent of pituitary growth hormone (GH). However, while fetal development is thought to reflect a *growth-without-GH* syndrome (Geffner, 1996), GH and GH mRNA are present in extrapituitary tissues of murine (Pantaleon *et al.*, 1997), piscine (Yang *et al.*, 1999) and avian (Harvey *et al.*, 2000a) embryos. It is therefore possible that extrapituitary GH acts as a paracrine or autocrine growth factor during early embryogenesis (Harvey *et al.*, 1997), especially as the distribution of GH overlaps the distribution of the GH receptor (Pantaleon *et al.*, 1997; Harvey *et al.*, 2000a) and as GH antibodies block the stimulatory effect of GH on the uptake of amino acids by murine embryos (Pantaleon *et al.*, 1997). The widespread distribution of GH in peripheral tissues of early chick embryos (Harvey *et al.*, 2000a) is also consistent with the presence of GH in neural, immune, reproductive, digestive and respiratory systems of neonates and adults (Wu *et al.*, 1996; Harvey and Hull, 1997; Tresguerres *et al.*, 1999; Allen *et al.*, 2000). It is therefore possible that other pituitary hormones in extrapituitary sites may similarly be expressed prior to the differentiation of the pituitary gland and its cell-types. Indeed, luteinizing hormone (LH)-immunoreactivity has been detected in the trachea, lung, esophagus and stomach of ED3-ED7 chick embryos, prior to the differentiation of pituitary gonadotropes on ED8 (Shirasawa *et al.*, 1996).

Thyrotropes appear ontogenically prior to somatotroph differentiation in mammals (Burrows *et al.*, 1999) and birds (Porter, 1997). The expression of thyrotropin

(βTSH) is not, however, confined to these cells in neonatal or adult mammals, since it also occurs in the brain (eg. Hojvat *et al.*, 1982a,b; 1985; DeVito *et al.*, 1985), in immune tissues (Smith *et al.*, 1983; Harbour *et al.*, 1989; Peele *et al.*, 1993; Bodey *et al.*, 2000) and in the gut (Wang *et al.*, 1997) and placenta (Harada and Hershman, 1978). The possibility that βTSH may be produced in extrapituitary tissues of the chick during early embryogenesis was therefore investigated in the present study, especially as βTSH- and GH- secreting cells are thought to be derived from the same cellular lineage, and as both βTSH and GH gene expression is thought to be dependent on the Pit-1 transcription factor (Burrows *et al.*, 1999).

II. MATERIALS AND METHODS

Tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit were incubated at 37.5 °C in humidified air. The eggs were turned one quarter of a revolution each day during incubation. At embryonic day (ED) 7 (stage 31) (Hamburger and Hamilton, 1951) the embryos were collected into phosphate-buffered saline (PBS, pH 7.4). Rathke's pouch is present at this stage of the 21 day incubation period, and extrapituitary GH-immunoreactivity is widespread in central and peripheral tissues of these embryos (Harvey *et al.*, 2000a).

Immunohistochemistry

Tissues were fixed in freshly prepared Carnoy's (60% v/v ethanol, 30% v/v chloroform, 10% v/v acetic acid) overnight at 4 °C. They were then dehydrated in a

graded series of ethanol (50%, 15-30 min; 70%, 30-60 min; 95%, 30-120 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax for 24-48 h at 60 °C, under vacuum. Serial (8 μm) sections were taken using a microtome and mounted on charged slides (Fisher Scientific). Transverse sections were taken through the head, and at the level of the wingbud, lung, heart, liver and mesonephros. Immunocytochemical staining was performed using the avidin-biotin-peroxidase (ABC) (Hsu *et al.*, 1981) method and commercial reagents (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with specific polyclonal antisera raised in rabbits against native chicken (c) GH (cGH1; Harvey & Scanes, 1977) or rat βTSH (βTSH; NIDDK-anti-rBeta βTSH-IC-I, AFP-1274789 (rabbit), Bethesda, Maryland, USA). Both antibodies were diluted in PBS (αcGH-1 at 1:1000; βTSH at 1:500) and incubations were overnight at room temperature. After incubation the sections were washed 3 times for 15 min in PBS and then incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (I_gG) (Vector 1:500). The sections were then washed in PBS and incubated in ABC reagent for 1 h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate diaminobenzidine tetrahydrochloride (DAB) (Sigma), which resulted in a brown precipitate. The specificity of staining was determined by preabsorbing the primary antibodies with recombinant cGH (Amgen, Thousand Oaks, CA, USA; 1 mg/ml) or rat βTSH (Bachem, Torrance, CA, USA; 1 mg/ml) for 1 h prior to section incubation. Antibody specificity was also demonstrated by the localization of GH-immunoreactivity in the caudal lobe of adult chicken pituitary glands (Fig 2.1A) and the localization of βTSH-immunoreactivity in the cephalic lobe (Fig 2.1B). Non-specific

staining was determined by replacing the primary antibodies with pre-immune rabbit serum or with PBS. Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ontario, Canada) mounted on an Olympus Bx40 microscope.

III. RESULTS

Immunoreactivity in the head

Within the head, GH-immunoreactivity was widespread, but most intense in neural tissue. Strong GH labeling was found in the anterior (Fig 2.1C) and posterior (Fig 2.2E) diencephalon. Within the diencephalon, the infundibulum was stained (Fig 2.1C), as were ependymal and subependymal cells lining the diocoele (Fig 2.1C; 2.2E). The GH staining in the diencephalon was in discrete layers of cells that were separated by concentric layers of unstained cells. GH-immunoreactivity was also present in adjacent accessory ganglia and in the trigeminal nerve (Fig 2.1C). Intense GH-immunoreactivity was also present in some, but not all, ependymal cells in the mesencephalon (Fig 2.2A, C). The mantle layer of the mesencephalon was also strongly labeled for GH-immunoreactivity (Fig 2.2C). βTSH-immunoreactivity was also present in ependymal cells of the diencephalon (Fig 2.1D) and mesencephalon (Fig 2.2B, D), but it was not in the mesencephalon mantle (Fig 2.2D), or anterior diencephalon (Fig 2.1D). The accessory ganglia and trigeminal nerve were also devoid of βTSH staining (Fig 2.1D, H), although the otic vesicle was strongly stained (Fig 2.2B).

In addition to the brain, faint GH- and βTSH staining was also present in the large (lateral rectus) muscles below the eyes (Fig 2.1C, D). βTSH-immunoreactivity was also

present in the outer epidermis (Fig 2.2B, F) and in endothelial cells lining the ethmoidal arteries (Fig 2.1D). These tissues were, however, devoid of GH staining (Fig 2.1C; 2.2A, E). In contrast, GH-immunoreactivity was present in the dura mater (Fig 2.2E), which lacked βTSH-staining (Fig 2.2F).

Rathke's pouch, the primordial pituitary gland, was devoid of specific GH or βTSH staining (Fig 2.1E, F). Control sections incubated with preabsorbed antisera or with NRS or PBS were also completely unstained (data not shown).

Immunoreactivity in the trunk

The outer epidermal cells of the trunk were again stained for βTSH but not for GH (Fig 2.3A, B). Intense GH-immunoreactivity was, however, present in the ventral and dorsal horns of the spinal cord and was particularly strong in the outer marginal layer and in the basal plate mantle (Fig 2.3A). In contrast, βTSH-immunoreactivity was restricted to ependymal cells lining the spinal canal (Fig 2.3B), which were devoid of GH-immunoreactivity (Fig 2.3A). Strong GH staining was also present in the dorsal and ventral root ganglia (Fig 2.3A), both of which lacked βTSH staining (Fig 2.3B).

In non-neural tissues, both GH- and βTSH- staining was present in the crop (Fig 2.3C, E). GH-immunoreactivity in this tissue was restricted to a single layer of endodermal cells lining the alimentary canal, whereas βTSH-immunoreactivity was more widespread, although not in every cell (Fig 2.3E). In the crop TSH-immunoreactivity was present in most adluminal cells and in cells on the basement membrane, with a layer of unstained cells between them (Fig 2.3E).

Within the developing lungs, βTSH-immunoreactivity was present in the cells lining the bronchial ducts (Fig 2.3F) (in adluminal cells and cells on the basement membrane) and in adluminal cells lining pleural cavities (Fig 2.4B; 2.5B, D). It was similarly present in cells lining the pericardial and hepatic cavities (Fig 2.4B; 2.5G). GH-immunoreactivity in contrast, was not present in these tissues (Fig 2.3D; 2.4A; 2.5C), but was intense in the surrounding intercostal muscle of the body wall (Fig 2.4A, C), which lacked βTSH staining (Fig 2.4B and E). Although the atria and ventricles lacked specific GH- or TSH-staining, squamous cells in the atrioventricular sulcus were stained for βTSH (Fig 2.4F), but not for GH (Fig 2.4D).

Immunoreactivity for GH was also intense in some cells in the Müllerian duct (Fig 2.5A), which lacked βTSH staining (Fig 2.5B). In the liver, hepatocytes were stained for both GH (Fig 2.5E) and βTSH (Fig 2.5F) immunoreactivity, in contrast to unstained erythrocytes within this tissue (Fig 2.5E, F). Intense staining for GH-immunoreactivity was also present in the right and left vagal nerve (Fig 2.5G), both of which lacked βTSH-immunoreactivity (Fig 2.5H). Control trunk sections incubated with preabsorbed antisera or with NRS or PBS were completely unstained (data not shown).

IV. DISCUSSION

These results clearly show, for the first time, the presence of βTSH-immunoreactivity in central and peripheral tissues of ED 7 chick embryos, prior to the differentiation of pituitary thyrotrophs. They also show that βTSH- and GH-immunoreactive cells are differentially located within embryonic tissues.

In the developing brain, βTSH-immunoreactivity was located in ependymal layers of the mesencephalon, in scattered cells within the trigeminal ganglia and in the otic vesicle. Although βTSH immunoreactivity has previously been detected by radioimmunoassay in rodent (Otteweller and Hedge, 1982; Hojvat *et al.*, 1982a; De Vito *et al.*, 1985; De Vito, 1989) and monkey (Hojvat *et al.*, 1982b) brains, its cellular location has not been shown. The predominately hypothalamic concentration of βTSH-immunoreactivity in the mammalian brain differs, however, from its cellular distribution in the embryonic chick brain. Since this immunoreactivity is present in the chick brain prior to the differentiation of pituitary thyrotrophs, it is likely to reflect *de novo* synthesis rather than sequestration from peripheral blood. The demonstration that the concentration of βTSH in the rat brain increases after colchicine blockade of axonal transport (De Vito, 1989) supports this view. This possibility is also supported by the pituitary-independent changes in brain-βTSH concentration that occur in rats diurnally (Ottenweller and Hedge, 1982), with growth (Emanuele *et al.*, 1985) and with endocrine manipulations (Hojvat *et al.*, 1985; De Vito *et al.*, 1985). The ontogeny of brain βTSH in the chick prior to the ontogeny of pituitary βTSH is in agreement with the earlier onset of βTSH immunoreactivity in the brains of fetal rats (Hojvat *et al.*, 1982).

The presence of βTSH receptors in the human brain (Labudova *et al.*, 1999) suggests roles for βTSH in neural function, especially as increased receptor abundance has been correlated with neurodegenerative disease and programmed cell death (Labudova *et al.*, 1999). Roles of βTSH in the chick brain are, as yet, unknown.

In the brain the localization of βTSH was principally in ependymal and sub-ependymal layers surrounding the mesocoele. It was also restricted to ependymal cells in

the spinal cord. In the periphery, βTSH immunoreactivity was similarly found in cellular layers surrounding body cavities (eg. in pericardial endoderm surrounding pericardial space; in the crop, surrounding the alimentary canal, in bronchial endoderm surrounding bronchial ducts and the pleural cavity). The adluminal location of βTSH immunoreactivity in the chick embryo may suggest transport or secretory roles for βTSH at these tissue interfaces, although roles for βTSH in these tissues are unknown. βTSH-immunoreactivity has previously been detected in the gut of adult rats (Wang *et al.*, 1997), but not in other embryonic tissues.

In addition to being produced in early chick embryos, βTSH is likely to have biological activity during early chick embryogenesis. Although receptors for βTSH have yet to be demonstrated in the chick embryo, the ability of exogenous (mammalian) βTSH to bind to the medullary cords of early chick embryonic (ED 6.5 – ED 11.5) ovaries (Woods *et al.*, 1991) and to embryonic (ED 8 – ED 15) Sertoli cells (Shahin *et al.*, 1982a,b) suggests actions for βTSH in the early embryo. Indeed, in early chick embryogenesis, βTSH promotes the migration of germ cells to the gonads (Shahin *et al.*, 1982b), the growth and differentiation of the gonad (Csaba *et al.*, 1980; Shahin *et al.*, 1982a,b) and the mitosis of the oocyte (Csaba *et al.*, 1980).

In addition to these extrathyroidal tissues, βTSH binding to "prefollicular" thyroid cells has also been determined in early (ED 5.5) chick embryos (Thommes *et al.*, 1992). The extrathyroidal actions of βTSH may, however, occur ontogenically prior to its thyrotrophic action, since βTSH-induced thyroid function has been demonstrated only in late-stage (ED 15-ED 21) chick embryos (Thommes and Hylka, 1978; Iqbal *et al.*, 1987;

Kühn *et al.*, 1988), when serum T_3 and T_4 levels are first detectable (Gregory *et al.*, 1998).

The ontogeny of extrapituitary βTSH in the chick embryo also appears to occur prior to its ontogeny in pituitary thyrotrophs. At ED 7, intense βTSH immunoreactivity was present in extrapituitary tissues of the chick embryo, although Rathke's pouch was devoid of βTSH staining. This suggests βTSH synthesis does not occur in the pituitary analogue during early ontogeny. This possibility is supported by the absence of βTSH mRNA in the pituitary glands of ED 11 embryos and by its minimal presence in the pituitary glands of ED 13 embryos (Gregory *et al.*, 1998). Although Thommes *et al.*, (1983) found immunoreactive βTSH in Rathke's pouch at ED 6.5; these cells were of low abundance until ED 11.5, when the caudal and cephalic lobes of the adenohypophysis were readily discernable. It is therefore likely that pituitary βTSH synthesis is minimal until the ontogenic differentiation of the thyrotroph cells.

The immunoreactivity for βTSH in peripheral tissues of the chick embryo was primarily in epithelial and endothelial cells surrounding body cavities or ductal systems. This luminal location is similar to the presence of LH-immunoreactivity in luminal cells of the esophagus, stomach, trachea and bronchi of ED 3-ED 7 chick embryos (Shirasawa *et al.*, 1996). It is therefore possible that these cells are derived from a similar glycoprotein hormone lineage. The βTSH immunoreactivity detected in the present study is not, however, due to cross-reactivity with LH, since the antibodies used have strict specificities and because the βTSH immunoreactivity is also in tissues devoid of LH immunoreactivity (eg. the brain and limb bud). The occurrence of βTSH and LH in the lung is, nevertheless, of interest, since other endocrine cells have been identified in the

chicken lung (Walsh and McLelland, 1978; Lopez *et al.*, 1983; Cook *et al.*, 1986), in which bombesin and somatostatin have been located (Adriaensen *et al.*, 1994). The location of these peptides differs, however, from the locations of βTSH (this study) and LH (Shirasawa *et al.*, 1996), since they were distributed on the basement membrane of the epithelium, in cells lacking direct contact with the luminal surface. It is also of interest that chorionic gonadotropin has been demonstrated in human fetal lungs (Yoshimoto *et al.*, 1977) and in benign lung neoplasms (Fukayama *et al.*, 1986).

The occurrence of GH-immunoreactivity in chick (Harvey *et al.*, 1997; 2000a) and murine (Pantaleon *et al.*, 1997) embryos prior to the differentiation of the pituitary gland is now well established. GH-immunoreactivity was present in the embryonic chick brain, as expected from the immunocytochemical distribution of GH in the metencephalon and diencephalon of adult turkeys and Ring doves (Ramesh *et al.*, 2000), the presence of GH-immunoreactive proteins in the brains of adult chickens (Render *et al.*, 1995) and the neonatal expression of the GH gene in the chicken brain (Render *et al.*, 1995). The ontogeny of neural GH therefore occurs in chickens prior to the ontogeny of pituitary somatotrophs (at ED 12-14, Jozsa *et al.*, 1979; Porter, 1997), as also observed in rats (Hojvat *et al.*, 1982b).

In addition to the brain, GH-immunoreactivity was also located in the white matter of the spinal cord (in the marginal layer and the basal plate mantle) and in dorsal- and ventral root ganglia of the chick embryo. It was also present in the trigeminal nerve and in the vagal nerve. A more widespread distribution of GH-immunoreactivity was previously observed in the neural tube of ED 3 embryos (Harvey *et al.*, 2000a), suggesting developmental changes occur in the production or action of GH in this neural

tissue. As GH is obligatory for normal neural development and neural function (reviewed by Harvey *et al.*, 1997), the results of these studies suggest local actions for GH in these neural sites.

In the periphery, strong GH-immunoreactivity was present in the intracostal muscles. Although this is a novel finding, the presence of GH-immunoreactivity in other peripheral tissues of ED 7 chick embryos (liver, Müllerian duct, wingbud chondrocytes) has been shown previously (Harvey *et al.*, 2000a). The absence of GH staining in the embryonic heart (Fig 2.4A) contrasts with our previous finding of GH-immunoreactivity in the atria of ED 7 embryos (Harvey *et al.*, 2000a), although as this immunoreactivity was lost by ED 8 (Harvey *et al.*, 2000a), this may reflect small differences between the embryos in their stage of development.

The presence of GH-immunoreactivity in the liver is of interest, since the liver is rich in biotin. The GH staining in the liver is not, however, an artifact of endogenous biotin in the ABC immunocytochemical technique (Polak and Van Noorden, 1997), since no staining was seen in the preabsorbed, NRS or PBS controls. Moreover, although DAB may stain non-specifically to hemoglobin in erythrocytes, in which endogenous peroxidase activity could cause a non-specific reaction (Van Bogaert *et al.*, 1981), the GH staining in hepatocytes contrasts with the absence of staining in red blood corpuscles in the liver (Fig 2.5E, F). The presence of GH in the embryonic liver prior to somatotroph differentiation therefore suggests it is not just a target site of GH action, but also a site of GH synthesis or sequestration in early embryos.

The distribution of GH-immunoreactivity in the ED 7 chick embryo was clearly very different from the distribution of βTSH. Although GH- and βTSH-

immunoreactivity were sometimes found in the same tissues (eg. in the brain, spinal cord and lung) they were in different cellular locations, as also observed in the anterior pituitary gland. Extrapituitary GH- and βTSH-cells are thus likely to have a different cellular lineage, whereas pituitary thyrotropes and somatotrophs in rats are thought to be derived from the same lineage (Burrows *et al.*, 1999). Indeed, thyrotropes and somatotropes are both absent in Snell dwarf mice, reflecting the lack of the Pit-1 transcription factor that is normally required for the differentiation of these cell-types.

However, βTSH-positive cells that fail to express Pit-1 have been characterized in the pars tuberalis of the rat pituitary gland (Sakai *et al.*, 1999), demonstrating the existence of heterogeneous populations of βTSH cell types. The Pit-1 dependence of pituitary and extrapituitary βTSH in embryonic or neonatal chicks is currently unknown, although GH expression in pituitary somatotrophs is thought to be Pit-1 dependent (Harvey *et al.*, 2000b).

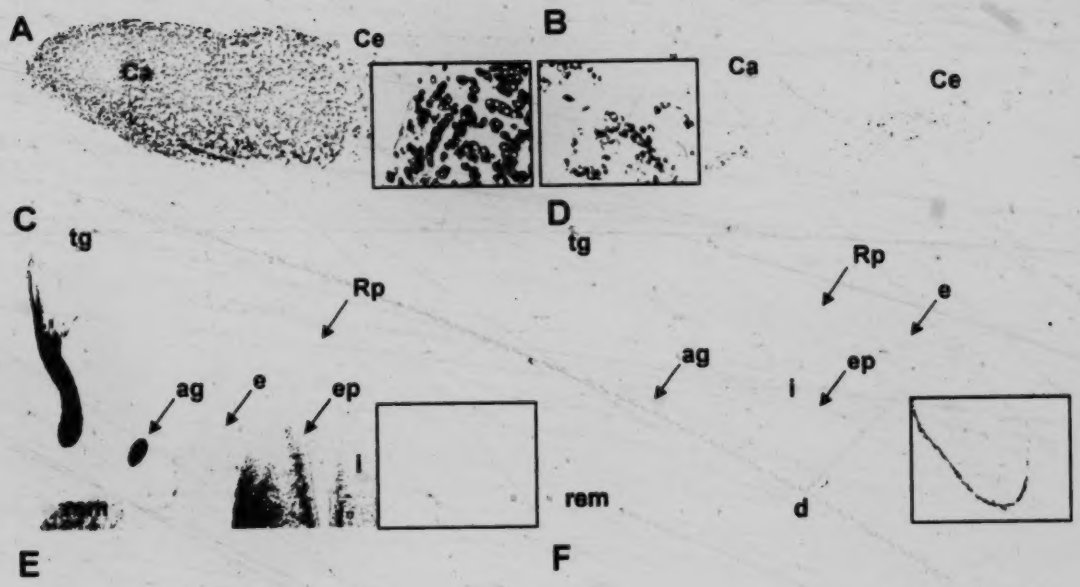
In summary, these results demonstrate the presence of GH- and βTSH-immunoreactive cells in extrapituitary tissues of early chick embryos prior to pituitary differentiation and show a differential distribution of these cell types.

Fig 2.1 Immunocytochemical localization of GH and βTSH.

Immunocytochemical localization of GH and βTSH in the adult chicken pituitary gland. (A) Localization of GH-immunoreactivity in the caudal lobe (Ca) of the adult chicken pituitary gland, (B) Localization of βTSH-immunoreactivity in the cephalic lobe (Ce) of the adult chicken pituitary gland. Magnification x100. The insets show higher magnifications (x1000) of the caudal and cephalic lobes.

Localization of GH-immunoreactivity in the head of a chick embryo at embryonic day (ED) 7. (C) GH-immunoreactivity is present in the diencephalon (d), infundibulum (i), trigeminal nerve (tg), accessory ganglia (ag), and the lateral rectus muscle (rem), but is not present in Rathke's pouch (Rp) or the endothelial cells of the ethmoidal artery (e). Magnification x100. The inset shows a higher magnification (x1000) of the ethmoidal artery. (E) Higher magnification (x1000) of Rathke's pouch. (G) Higher magnification (x1000) of the trigeminal nerve.

Localization of βTSH-immunoreactivity in the head of an ED 7 chick embryo. (D) Specific βTSH staining is present in the ependymal cells (ep) of the diencephalon (d) and in the endothelial cells of the ethmoidal artery (e). The mantle layers of the diencephalon (d), the trigeminal nerve (tg), accessory ganglia (ag) and Rathke's pouch (Rp) are unstained. Magnification x100. The inset shows a higher magnification (x1000) of the endothelial cells in the ethmoidal artery. (F) Higher magnification (x1000) of Rathke's pouch. (H) Higher magnification (x1000) of the trigeminal nerve.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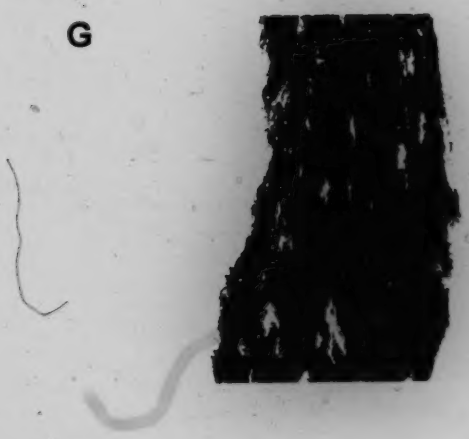
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Fig 2.2 GH- and βTSH-immunoreactivity in the head of 7 day-old chick embryos. (A) GH-staining is shown in the ependymal cells (ep) of the mesencephalon (m), but not in the otic vesicle (ov). Magnification x100. The insert shows a higher magnification (x1000) of the otic vesicle. (B) βTSH staining in the ependymal cells (ep) of the mesencephalon (m). The inset shows a higher magnification (x1000) of the otic vesicle (ov). (C) GH-immunoreactivity in the mantle of the mesencephalon (m). Note the absence of staining in the ependymal cells (ep). Magnification x100. The inset is a higher magnification (x1000) of the mantle at the marked (+) area. (D) βTSH-immunoreactivity in the ependymal cells (ep) of the mesencephalon (m). Magnification x100. The inset is a higher magnification (x1000) of the marked (+) area. (E) GH-staining in the mantle region of the diencephalon (d) and in the dura mater (dm). Note the absence of staining in the epidermis (ed). Magnification x100. The inset shows a higher magnification (x1000) of the marked (+) area. (F) βTSH-staining in the mantle region of the diencephalon (d) and in the epidermis (ed). Magnification x100. Note the restriction of staining to the ependymal cells (ep) lining the diocele. Magnification x100. The inset shows a higher magnification (x1000) of the marked (+) area.

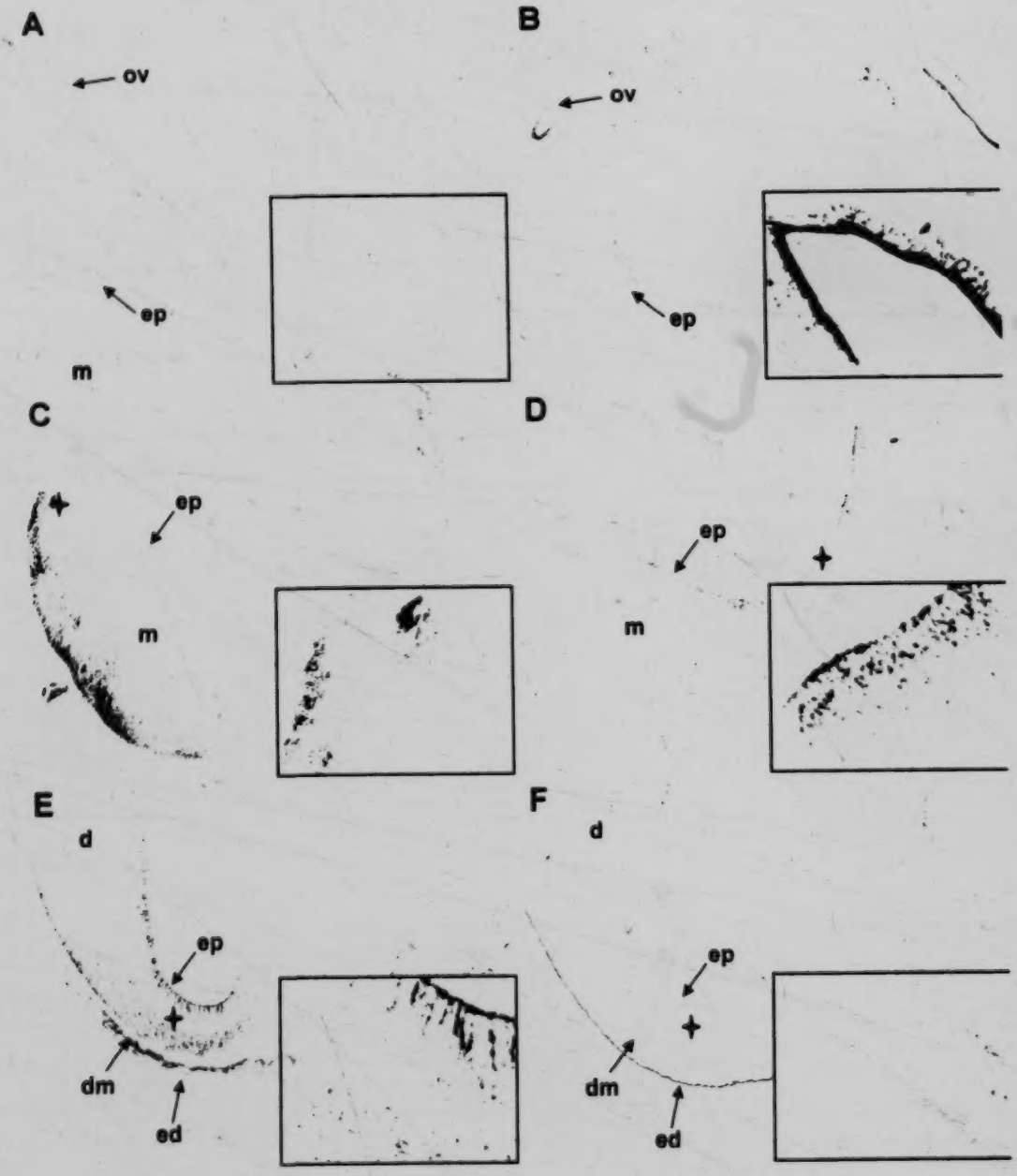
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Fig 2.3 GH- and βTSH-immunoreactivity in peripheral tissues of a 7-day-old chick embryo. (A) GH-staining in the mantle (m) and marginal layer (ml) of the spinal cord (sc) and in the dorsal- (d) and ventral- (v) root ganglia. Magnification x100. The inset shows a higher magnification (x1000) of the mantle and marginal layer. (B) βTSH-staining in the ependymal (e) cells of the spinal cord and in the epidermis (arrow). Note the absence of staining in other areas of the spinal cord and the lack of staining in the dorsal- (d) and ventral- (v) root ganglia. Magnification x100. The inset shows a higher magnification (x1000) of the ependymal cells surrounding the spinal canal. (C) GH-immunoreactivity in endothelial cells lining the crop (arrowed). Magnification x1000. (D) Absence of GH-staining in a bronchus of the developing lung. Magnification x1000. (E) βTSH-immunoreactivity in the crop. Magnification x1000. (F) βTSH-immunoreactivity in a bronchus of the developing lung. Magnification x1000.

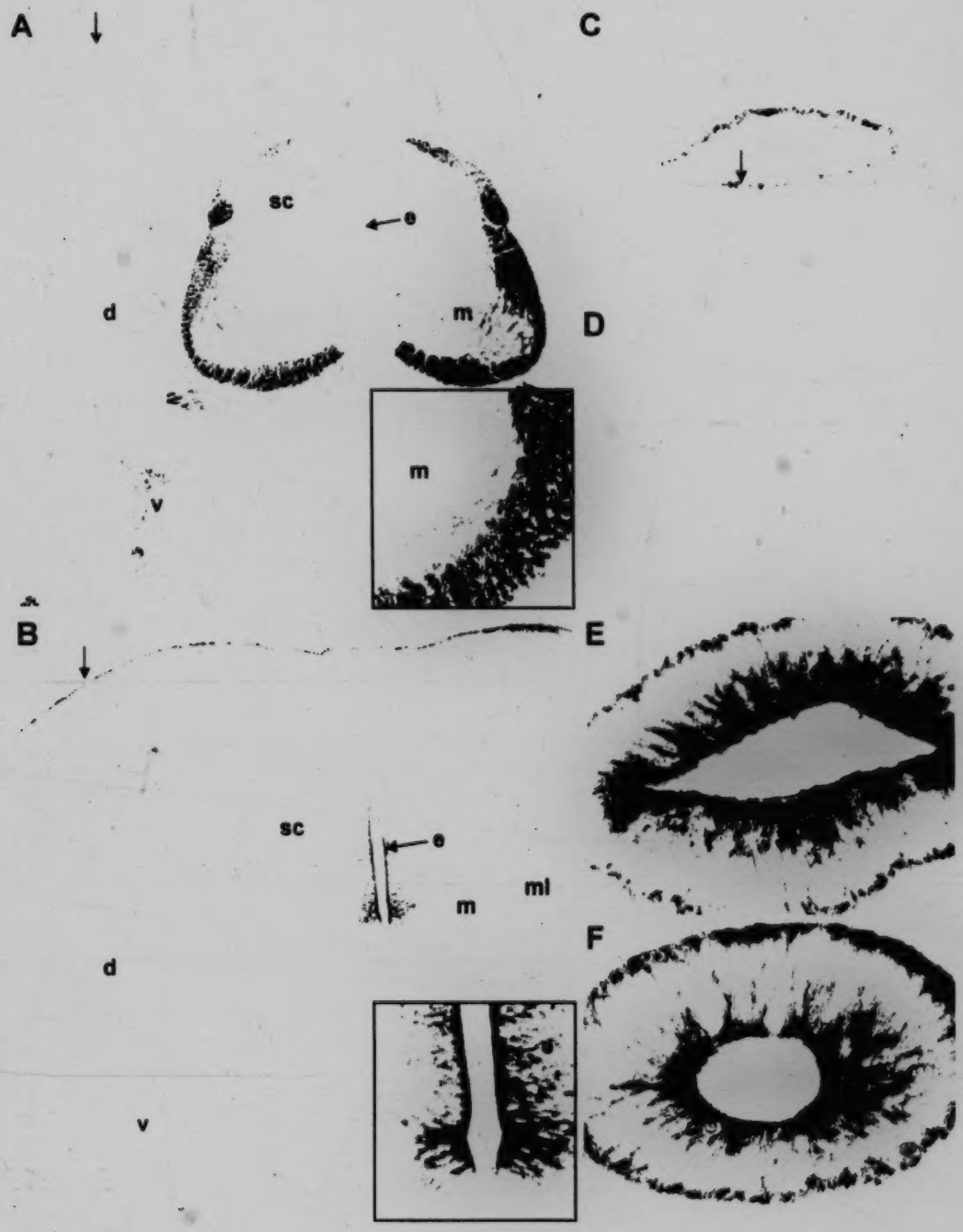
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Fig 2.4 GH-and βTSH-immunoreactivity in peripheral tissues of a 7-day-old chick embryo. (A) GH-staining in intracostal muscles (i) between developing ribs, surrounding the body cavity. Note the absence of GH-staining in the bronchi (b), dorsal aorta (d), atria (a) and the atrioventricular sulcus (avs). Magnification x100. (C) A higher magnification (x1000) of the intracostal muscle (i). (D) A higher magnification (x1000) of the atrioventricular sulcus. (B) βTSH-staining in the crop (c), and bronchi (b), and in cells lining pleural cavities (pc) and the pericardial space (ps) and in the atrioventricular sulcus (avs). Note the absence of βTSH-staining in the intracostal muscle (i). Magnification x100. (E) Higher magnification (x1000) of the intracostal muscle (i), and pericardial space. (F) Higher magnification (x1000) of the atrioventricular sulcus.

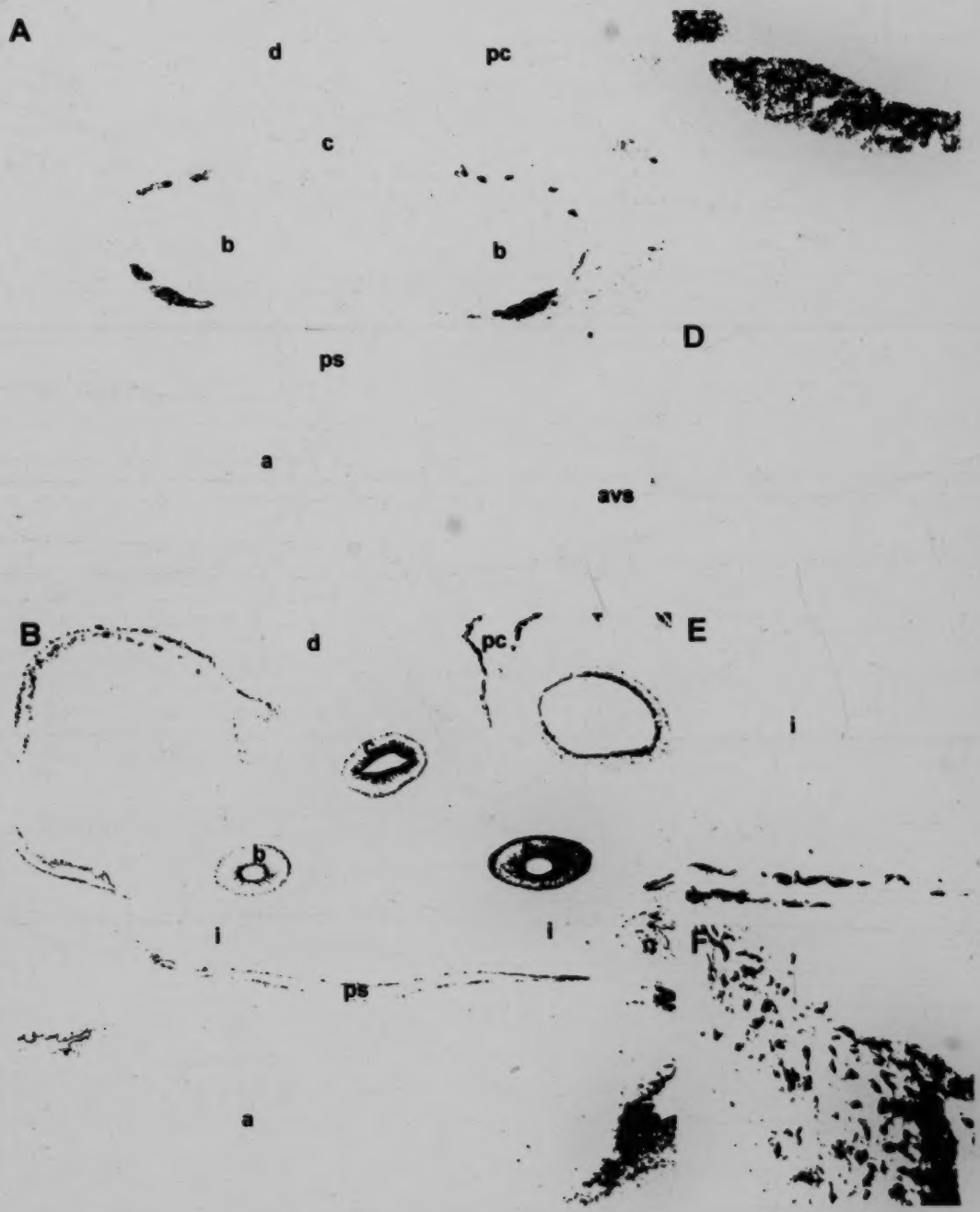
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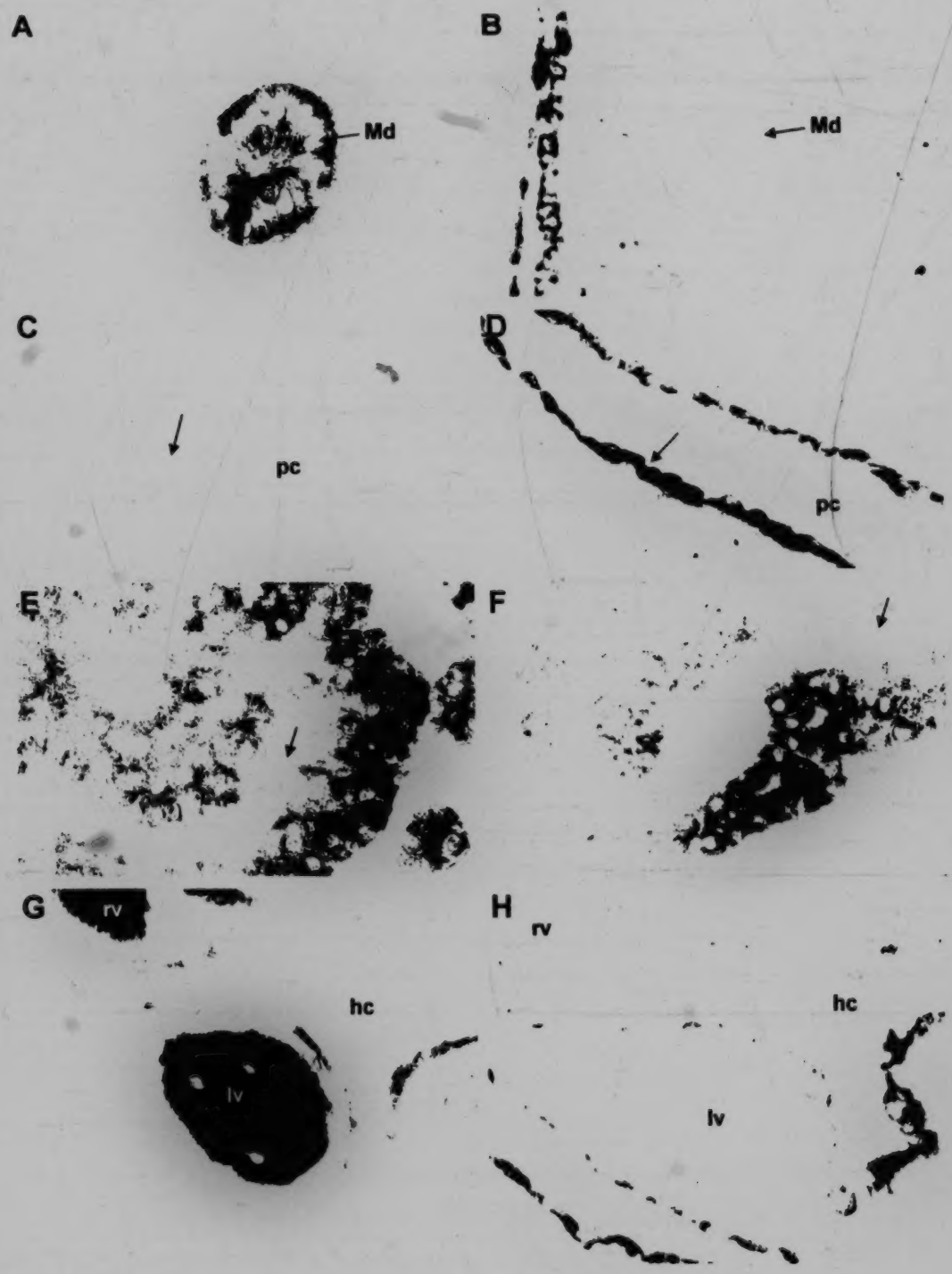
Fig 2.5 GH- and βTSH-immunoreactivity in peripheral tissues of 7-day-old chick embryos. GH- staining is present in (A) the Müllerian duct (md), (E) in liver hepatocytes and (G) the right (rv) and left (lv) vagal nerve, but not in (C) cells lining the pleural cavity (pc) (arrow), nor (E) in liver erythrocytes (arrow). βTSH-staining is in cells lining the pleural cavity (pc) (B,D) and hepatic cavity (hc) (H) and in liver hepatocytes (F), but not (B) in the Müllerian duct (md), (F) liver erythrocytes (arrow), or (H) vagal nerves. Magnification x1000.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Chapter 3

Extrapituitary TSH in Early Chick Embryos: Pit-1 Dependence?

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I. INTRODUCTION

Thyrotropin-stimulating hormone (TSH) is primarily produced in pituitary thyrotrophs, although it is also found in extrapituitary sites. TSH immunoreactivity is, for instance, present in the rat brain (Hojvat *et al.*, 1982a,b, 1985), particularly in the hypothalamus (De Vito *et al.*, 1985, 1989). The TSH gene is also thought to be expressed in the human immune system (Smith *et al.*, 1983; Harbour *et al.*, 1989; Peele *et al.*, 1993; Bodey *et al.*, 2000), gut (Wang *et al.*, 1997) and placenta (Harada and Hershman, 1978). It was also recently detected in embryonic chicks, in discrete cells in the developing brain (in ependymal cells lining the diocoele and mesocoele and in the lining of the otic vesicle), spinal cord (in ependymal cells), liver (in hepatocytes), lungs (in the linings of the bronchi), gut (in the linings of the proventriculus) and cardiopulmonary system (in cells in the adluminal linings of the pleural and pericardial cavities) (Murphy and Harvey, 2001). In the pituitary, the expression of the TSH gene is thought to be dependent upon the pituitary-specific transcription factor, Pit-1 (Burrows *et al.*, 1999). The presence of TSH in extrapituitary tissues therefore suggests a widespread distribution of Pit-1 or that extrapituitary TSH is independent of Pit-1 regulation. This possibility has been assessed in the present study, by comparing the distribution of TSH and Pit-1 in central and peripheral tissues of early chick embryos, prior to the ontogenic differentiation of the pituitary gland.

II. MATERIALS AND METHODS

Tissues

Fertile White Leghorn eggs from the University of Alberta Poultry Unit were incubated at 38 °C in humidified air. At embryonic day (ED) 7 (stage 31) (Hamburger and Hamilton, 1951), the embryos were collected into phosphate-buffered saline (PBS, pH 7.4). Rathke's pouch, the pituitary precursor, is present at this stage of the 21-day incubation period, and extrapituitary TSH immunoreactivity is widespread in central and peripheral tissues of these embryos (Murphy and Harvey, 2001).

Immunocytochemistry

Tissues were fixed in freshly prepared Carnoy's (60% v/v EtOH, 30% v/v chloroform, 10% v/v acetic acid) overnight at 4 °C. They were then dehydrated in a graded series of ethanol (50%, 15-30 min; 70%, 30-60 min; 95%, 30-120 min; 100%, 30-120 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 30 min. Tissues were then infiltrated with paraffin wax for 24-48 h at 60 °C, under vacuum. Serial (8 μm) transverse sections were taken using a microtome and mounted on charged slides (Fisher Scientific). Sections were taken through the head, and at the level of the wing bud, lung, heart, liver and mesonephros. Immunocytochemical staining was performed using the avidin-biotin-peroxidase (ABC) method (Hsu *et al.*, 1981) and commercial reagents (Vector Laboratories, Burlingame, CA, USA). Adjacent sections were incubated with specific polyclonal antisera raised in rabbits against rat βTSH (αβTSH; NIDDK-anti-rBeta TSH-IC-I, AFP-1274789 (rabbit), Bethesda, MD, USA) or human Pit-1 (αPit-1, #132; Voss *et al.*, 1991). Both antibodies were diluted in PBS (αβTSH at 1:500; αPit-1 at 1:200) and incubations were overnight at 4 °C. After incubation, the sections were washed three times for 15 min in PBS and then incubated

for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobulin G (I_gG) (Vector, 1:500). The sections were then washed in PBS and incubated in ABC reagents for 1 h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate, diaminobenzide tetrahydrochloride (DAB) (Sigma), which resulted in a brown or blue precipitate. The specificity of TSH staining was determined by preabsorbing the primary antibody with rat βTSH (Bachem, Torrance, CA, USA; 1 mg/ml) for 1 h prior to section incubation, which completely abolished all staining. Antibody specificity was also demonstrated by the localization of TSH-immunoreactivity in the cephalic lobe of adult chicken pituitary glands (Fig. 3.1A). Pit-1 immunoreactivity was present in both the cephalic and caudal lobes of the anterior pituitary (Fig. 3.1B), presumably reflecting the presence of growth hormone (GH) in the caudal lobe (Murphy and Harvey, 2001) and TSH and prolactin in the cephalic lobe (Mikami, 1983). Staining for βTSH was restricted to the cytoplasm of pituitary cells, whereas Pit-1 immunoreactivity was mostly nuclear. Replacing the primary antibodies with preimmune rabbit serum or with PBS abolished all staining. Digital images were collected using a SPOT Digital Microscope camera (Carsen Group, Markam, Ontario, Canada) mounted on an Olympus Bx40 microscope. Some of the staining for TSH was reported previously (Murphy and Harvey, 2001) and is presented here for comparison.

Western Analysis

Pooled ED 7 chick embryo tissues (heads, headless trunks, wingbuds and whole eyes) and adult chicken pituitary glands were placed in protease inhibitor solution (Hepes, $MgCl_2$, EDTA, EGTA, aprotinin, leupeptin and pepstatin) plus 1% v/v PMSF

and quickly homogenized using a polytron homogenizer (Brinkman Instruments, Westbury, NY, USA). Sample homogenates were centrifuged for 5 min, the supernatant collected and a protein assay was done to determine protein concentration. 50 µg of embryo tissue protein and 10 µg of pituitary protein were added to loading buffer (10% glycerol, 5% 2-β-mercaptoethanol, 2% SDS, 0.001% bromophenol blue; pH 6.8) plus 10% DTT and denatured at 70 °C for 5 min prior to loading. Proteins were separated by SDS-polyacrylamide gel (15% w/v) electrophoresis under reducing conditions. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) and transferred electrophoretically (100V for 1 h at 4 °C) to Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Non-specific binding sites were blocked by incubating the membrane with 5% skim milk in Tris buffered saline (25 nM TrisHCl, 0.5 M NaCl; pH 7.6) plus Tween® 20 (TBST) for 1 h at room temperature. Pit-1 immunoreactivity was detected using a polyclonal antibody raised in rabbits against human Pit-1 (αPit-1, #132; Voss *et al.*, 1991) diluted 1:50 in TBST/5% skim milk. Membranes were washed with TBST (3 x 10 min), incubated for 1 h at room temperature with a biotinylated goat anti-rabbit immunoglobulin G (I_gG) (Vector, 1:500), washed with TBST (3 x 10 min) and then incubated in ABC reagents (Vector) for 1 h at room temperature. After washing, blots were developed with an enhanced chemiluminescence detection system (ECL Kit, Amersham Pharmacia Biotech) and exposed to Kodak X-AR film (Kodak, Rochester, USA).

Reverse Transcription - Polymerase Chain Reaction

The presence of the Pit-1 gene was assessed using the reverse transcription polymerase chain reaction (RT-PCR). Total RNA from pooled ED 7 chick embryo tissues (heads, headless trunks, and wingbuds), adult chicken pituitary glands and muscle was isolated using the QIAGEN RNeasy Mini kit and protocol (QIAGEN, Mississauga, ON, Canada). The RNA was reverse transcribed in the presence of 10 pmol of the oligo-deoxythymine primer, Khu 14, 5'- GACTCGAGTCGACATCGTTTTTTTTTTTT TTTT-3', (DNA Core Facility, University of Alberta), 5x First-Strand Buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), excess deoxynucleotides (10 nM each deoxy-ATP, dCTP, dGTP and dTTP) (Invitrogen Life Technologies), the reverse transcription enzyme Superscript II (200 U, Invitrogen Life Technologies) and 0.1 M dithiothreitol (DTT). This was incubated for 1 h at 42 °C and inactivated for 10 min at 70 °C. The newly generated cDNA was diluted in 200 µl of double-distilled water. Transcribed pituitary RNA was used as a positive control and transcribed muscle RNA and RNA that was not transcribed (no Superscript II was added to the mixture) were used as negative controls.

The cDNA was amplified in the presence of oligonucleotide primers Pit-1(11), 5'-GCTCTAGACAGCAGGACTCTACTCT-3' and Pit-1 (12), 5'- GCTCTAGAGTCTGGT GCATTGGTGTGAA-3', (DNA Core Facility, University of Alberta) and Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The samples were then placed in a Techne thermal cycler (Techne Incorporated, Princeton, NJ, USA), denatured at 95 °C for 5 min, amplified for 35 cycles of denaturing (95 °C for 1 min), annealing (57 °C for 1 min) and extension (72 °C for 2 min), followed by a final

extension of 10 min at 72 °C. The amplified cDNA (10 µl) was electrophoresed on a 1.5% (w/v) agarose ethidium bromide gel and visualized with UV light.

Southern Blotting

Pit-1 cDNA amplified by PCR was transfered to a nylon membrane using the S&S TurboBlotter and Blotting Stack Assembly for Alkaline Transfers (Schleicher & Schuell, Keene, NH, USA). After transfer, the DNA was fixed to the membrane by baking at 80 °C for 30 min in a vacuum oven. The blot was prehybridized in 10 ml of DIG Easy Hyb hybridization buffer (Roche Applied Science, Basel, Switzerland) for 1 h at 50 °C. The DNA blot was then hybridized with 10 pmol digoxigenin (DIG) labeled Pit-1 RNA probe/ml hybridization buffer overnight at 50 °C. The hybridization buffer was poured off and the blot was washed 2 x 5 min in 2 x SSC (Roche Applied Science) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature, 2 x 5 min in 0.5 x SSC containing 0.1% SDS at 65 °C and 1 x 1 min in 0.3% (v/v) Tween® 20 in maleic acid buffer (10 mM maleic acid, 15 mM NaCl; pH 7.5) at room temperature. The blot was incubated in 1 x Blocking Reagent (Roche Applied Science) for 1 h with gentle shaking and then with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) diluted 1:5000 in 1 x Blocking Reagent. Colormetric detection of the DIG labeled probe was carried out using NBT/BCIP (Roche Applied Science).

III. RESULTS

Immunoreactivity in the head

Within the head, TSH immunoreactivity was present in the linings of the otic vesicle and in the ependymal and subependymal lining of the mesocoele (Fig. 3.1C). Pit-1 immunoreactivity was also present in these sites, but was most abundant in the cells and fibres in the trigeminal nerve (Fig. 3.1H), in which TSH staining was slight and restricted to scattered cells. Immunoreactivity for TSH was also present in ependymal and subependymal cells lining the anterior and posterior diencephalon (Fig. 3.2A, C, E) and in infundibular cells (Fig. 3.2A). It was also present in the epidermis (Fig. 3.2C) and in the endothelial cell lining of the ethmoidal artery (Fig. 3.2C). In marked contrast, staining for Pit-1 was not seen in any of these sites (Fig. 3.2B, D, F), or in Rathke's pouch (Fig. 3.2B), which was also devoid of TSH immunoreactivity (Fig. 3.2A).

Immunoreactivity in the trunk

The epidermal cells of the trunk were strongly immunoreactive for TSH (Fig. 3.3A), but not for Pit-1 (Fig. 3.3B). TSH immunoreactivity was also intense in the ependymal and subependymal cells of the spinal cord and weakly present in the dorsal root ganglia (Fig. 3.3A). In contrast, Pit-1 immunoreactivity was intense in the upper marginal layer of the spinal cord and abundant in the dorsal root ganglia (Fig. 3.3B). A single adluminal layer of spinal cord ependymal cells also had Pit-1 immunoreactivity (Fig. 3.3E), as did cells in the myotome (Fig. 3.3F). Slight TSH immunoreactivity was present in a small number of cells in the marginal layer of the spinal cord, although most were not immunreactive (Fig. 3.3C). TSH immunoactivity was also absent in the myotome (Fig. 3.3D).

At the level of the limb bud, TSH immunoreactivity was widespread and intense (Fig. 3.4A). It was particularly strong in a subepidermal layer of cells (Fig. 3.4C) and in pockets of the brachial nerve plexus (Fig. 3.4D), but it was also in the dorsal and ventral muscle blocks and in chondrocytes in the humerus (Fig. 3.4A). In marked contrast, there was no staining for Pit-1 in the limb bud (Fig. 3.4B, E, F). In the internal organs, heavy TSH immunostaining was present in the bronchi of the lungs (Fig. 3.5A) and in the proventriculus (crop) of the gastrointestinal tract (Fig. 3.5C). There was no Pit-1 immunoreactivity in the lung (Fig. 3.5B) and only a single layer of Pit-1 immunoreactive cells that lined the crop lumen (Fig. 3.5D). Similarly, while cells lining the pleural cavity had intense TSH immunoreactivity (Fig. 3.6A), these cells were completely devoid of Pit-1 immunostaining. In contrast, hepatocytes were strongly immunoreactive for both TSH and Pit-1 (Fig. 3.6C, D). No other peripheral tissues contained TSH or Pit-1 immunoreactivity.

Western Analysis

A Pit-1-like protein of approximately 33 kDa was detected using αPit-1 #132 in the adult chicken pituitary gland, ED 7 chick head, trunk, wingbud and whole eye (Fig. 3.9).

Reverse Transcription - Polymerase Chain Reaction

In the presence of oligonucleotide primers for pituitary Pit-1 cDNA (Pit-1 (11) and Pit-1 (12)), a moiety of approximately 279 bp was amplified from the adult chicken

pituitary gland as visualized with ethidium bromide staining (Fig. 3.8A). No bands were visible for ED7 chicken embryo tissues or the negative controls.

Southern Blotting

In the presence of a DIG labeled Pit-1 RNA probe, a moiety of approximately 279 bp was detected for amplified adult chicken pituitary and ED7 chick head, trunk and wingbud cDNA (Fig. 3.8B). No bands were visible in the negative controls.

IV. DISCUSSION

These results demonstrate, for the first time, the presence of Pit-1 immunoreactivity in pituitary and extrapituitary cells in the chicken. They also clearly show that Pit-1 immunoreactivity in the early chick embryo is present in tissues with and without TSH immunoreactivity. The extrapituitary expression of the TSH gene may thus be tissue-specific, and in some tissues appears to be Pit-1 independent.

This is the first demonstration of Pit-1 immunoreactivity in avian tissues, although the Pit-1 gene has been cloned and sequenced from chicken (Buys *et al.*, 1998; Tanaka *et al.*, 1999; Harvey *et al.*, 2000) and turkey (Wong *et al.*, 1992) pituitary glands. The most abundant transcript, Pit-1α, has sequence homology with mammalian Pit-1, but other avian-specific variants are also expressed, although in much smaller amounts (Tanaka *et al.*, 1999). It is therefore likely that the immunoreactivity detected in chicken tissues reflects the presence of Pit-1α or a very similar protein, especially as the antisera used has cross-reactivity with Pit-1-like proteins in more primitive vertebrates (Candiani and Pestarino, 1998a,b, 1999) and Western analysis revealed a protein of 33 kDa in

embryonic chick tissues, the same molecular weight as pituitary Pit-1 and mammalian Pit-1 (Harvey *et al.*, 2000). Indeed, the Pit-1 gene appears to have been conserved during phylogenetic evolution (Harvey *et al.*, 2000) and Pit-1-like proteins are even present in sponges (Seimiya *et al.*, 1997).

In the pituitary gland, Pit-1 is thought to regulate the transcription of the GH, prolactin and TSH genes (Harvey *et al.*, 2000). The somatotrophs in the caudal lobe are spatially separated from the thyrotrophs and lactotrophs in the cephalic lobe of the chicken pituitary gland (Mikami, 1983), and Pit-1 immunoreactivity was, accordingly, found in both the caudal and cephalic lobes of the adenohypophysis. In addition to regulating the pituitary expression of these genes, Pit-1 is thought to be obligatory for the phenotypic differentiation of the GH-, prolactin- and TSH-secreting cells (Harvey *et al.*, 2000). Indeed, these cells are thought to be derived from the same cellular lineage and are absent in Pit-1 deficient Snell dwarf mice (Burrows *et al.*, 1999). It is therefore of interest that Pit-1 immunoreactivity was not detected in Rathke's pouch, which was also devoid of TSH immunoreactivity at this stage of embryonic development (Fig. 3.2). The ontogeny of pituitary TSH expression in the chick embryo therefore appears to occur after the ontogeny of extrapituitary TSH production. The absence of βTSH mRNA in the pituitary glands of ED 11 embryos (Gregory *et al.*, 1998) supports this view. Thommes *et al.* (1983) previously found some immunoreactive βTSH in Rathke's pouch at ED 6.5, but these cells were of low abundance until ED 11.5, when the caudal and cephalic lobes of the adenohypophysis were readily discernable. It is therefore likely that pituitary TSH synthesis is minimal until the ontogenic differentiation of thyrotroph cells. The ontogeny of Pit-1 gene expression in the pituitary glands of embryonic chicks is similarly not

thought to occur until the second third of incubation (Geris *et al.*, 2002), even though extrapituitary Pit-1 mRNA is present in ED 2 embryos and the headless bodies of ED 6 and ED 8 embryos (Harvey *et al.*, 2000), and this study demonstrates Pit-1 mRNA in tissues of the ED 7 chick embryo (heads, headless and wingless trunks, wingbuds). Pit-1 is thus not pituitary-specific in its expression in embryonic chicks and its extrapituitary expression occurs before that in the pituitary, as also observed in rats (He *et al.*, 1989; Dolle *et al.*, 1990).

The so-called pituitary specificity of Pit-1 is based on early studies that indicated it was absent from the intestine, liver, lung, spleen, thyroid, thymus, pancreas, kidney, muscle, heart, testes, prostate, uterus, brain and neural retina, and was lacking in fibroblasts, hepatocytes and placental cells (reviewed by Harvey *et al.*, 2000). More recent studies have, however, indicated its presence in the rat (He *et al.*, 1989), mouse (Dolle *et al.*, 1990) and chicken (Yoshizato *et al.*, 1998) brain, as well as its presence in the placenta, spleen, thymus, bone marrow and kidney of mammals (Harvey *et al.*, 2000). The results of the present study extend the list of extrapituitary tissues that appear to express the Pit-1 gene. In addition to neural tissues, it was found in skin, muscle and bone, and was present in the liver, gastrointestinal tract and the cardiopulmonary system.

In some of these tissues (brain and spinal cord ependymal cells, neural cells in the otic vesicle, and liver hepatocytes), the distribution of Pit-1 immunoreactivity overlapped the distribution of immunoreactive TSH. This overlapping pattern of distribution may reflect a casual relationship between Pit-1 and TSH expression and indicates that extrapituitary TSH may be Pit-1 dependent. However, in other tissues (eg. the trigeminal nerve in the head and the marginal mantle layer of the spinal cord), Pit-1

immunoreactivity was intense, but TSH immunoreactivity was marginal. Conversely, other tissues (e.g. cells in the skin, blood vessels, limb bud, bronchus, proventriculus and cardiopleural cavities) had intense TSH staining but little, if any, Pit-1 immunoreactivity. The Pit-1 dependence of the TSH gene may therefore be tissue-specific. Sakai *et al.* (1999) similarly found that both Pit-1 and TSH were present in the rat pars distalis, but only TSH was present in the pars tuberalis.

The disparate distribution of Pit-1 and TSH in many extrapituitary tissues may thus indicate that it is not involved in extrapituitary TSH regulation. The disparate distribution of Pit-1 may, for instance, reflect its regulation of genes other than TSH, GH and prolactin, especially as it is known to be involved in the expression of the renin gene (Cantanzaro *et al.*, 1994; Gilbert *et al.*, 1994; Germain *et al.*, 1996) and the genes for the somatostatin receptor (Baumeister and Meyerhof, 1998), and the GH-releasing hormone receptor (Lin *et al.*, 1992; Gaylinn, 1999; Iguchi *et al.*, 1999; Miller *et al.*, 1999). Pit-1 binding sites have also been detected in the promoter regions of the gonadotropin-releasing hormone receptor (Fan *et al.*, 1995; Kakar, 1997), the thyrotropin-releasing hormone receptor, the secretogranin II gene (Jones *et al.*, 1996) and the somatolactin gene (Takayama *et al.*, 1991; Ono *et al.*, 1995). It is therefore possible that the presence of Pit-1 in extrapituitary tissues may reflect a variety of tissue-specific actions.

In summary, these results demonstrate that Pit-1 immunoreactivity is widespread in tissues of the chick embryo, although the expression of the pituitary TSH gene is thought to be Pit-1 dependent. The disparate location of Pit-1 and TSH immunoreactivities in these tissues suggests TSH expression in extrapituitary tissues may be Pit-1 independent.

Fig 3.1 Immunocytochemical localization of TSH and Pit-1. (A) Localization of TSH immunoreactivity in the cephalic (Ce) lobe of the adult chicken pituitary gland. (B) Localization of Pit-1 immunoreactivity in both the cephalic and caudal (Ca) lobes of the gland. Magnification x100. The insets show higher magnifications (x1000). (C) Localization of TSH immunoreactivity in the head of an ED 7 chick embryo. Staining is present in the ependymal and subependymal cells lining the mesocoele (mc) and in the otic vesicles (ov). Magnification x100. The inset shows a higher magnification (x1000) of the otic vesicles. (D) Localization of Pit-1 immunoreactivity in the head of the ED 7 chicken embryo. Staining is present in the otic vesicles and in the ependymal lining of the mesocoele. Magnification x100. The inset shows a higher magnification of the otic vesicle (x1000). (E) TSH immunoreactivity in ependymal mesencephalon (m). Magnification x100. (F) Pit-1 immunoreactivity in the trigeminal nerve (tg). (G) Higher magnification (x1000) of the TSH immunoreactivity in ependymal cells surrounding the mesocoele (boxed area in [E]). (H) Higher magnification (x1000) of the Pit-1 immunoreactivity in the trigeminal nerve (boxed area in [F]). TSH staining in (C), (D) and (E) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.

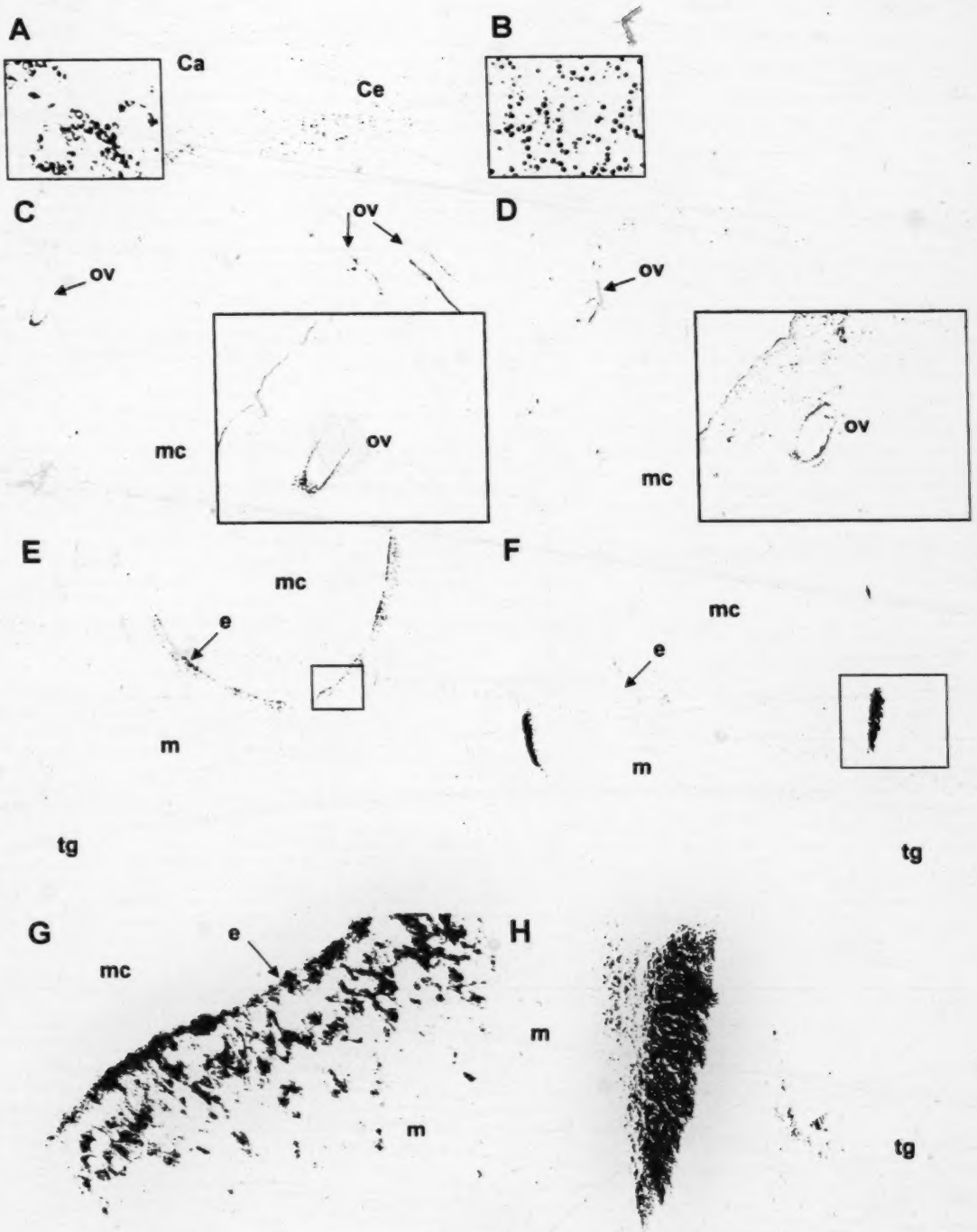
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Fig 3.2 TSH and Pit-1 immunostaining of the ED 7 chick embryo head. (A) TSH immunoreactivity is present in ependymal and subependymal cells in the infundibular (i) region of the diencephalon and in endothelial cells (arrow) of the ethmoid artery (bv). Rathke's pouch (Rp) is devoid of TSH staining. Magnification x100. (B) Pit-1 immunoreactivity is not present in a similar section of the ED7 embryo head. Magnification x100. (C) TSH immunoreactivity in ependymal cells (e) lining the diocoele (dc) in the mantle margin of the diencephalon (d) and in the outer epidermis (ep) (arrow). Magnification x100. The inset shown in (E) shows TSH immunoreactivity in ependymal cells in the mantle region of the diencephalon at a higher (x1000) magnification. (D) and (F) Pit-1 immunoreactivity is not present in similar sections of the ED 7 embryo head. Magnification x100 and x1000, respectively. TSH immunoreactivity in (A), (C) and (E) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.

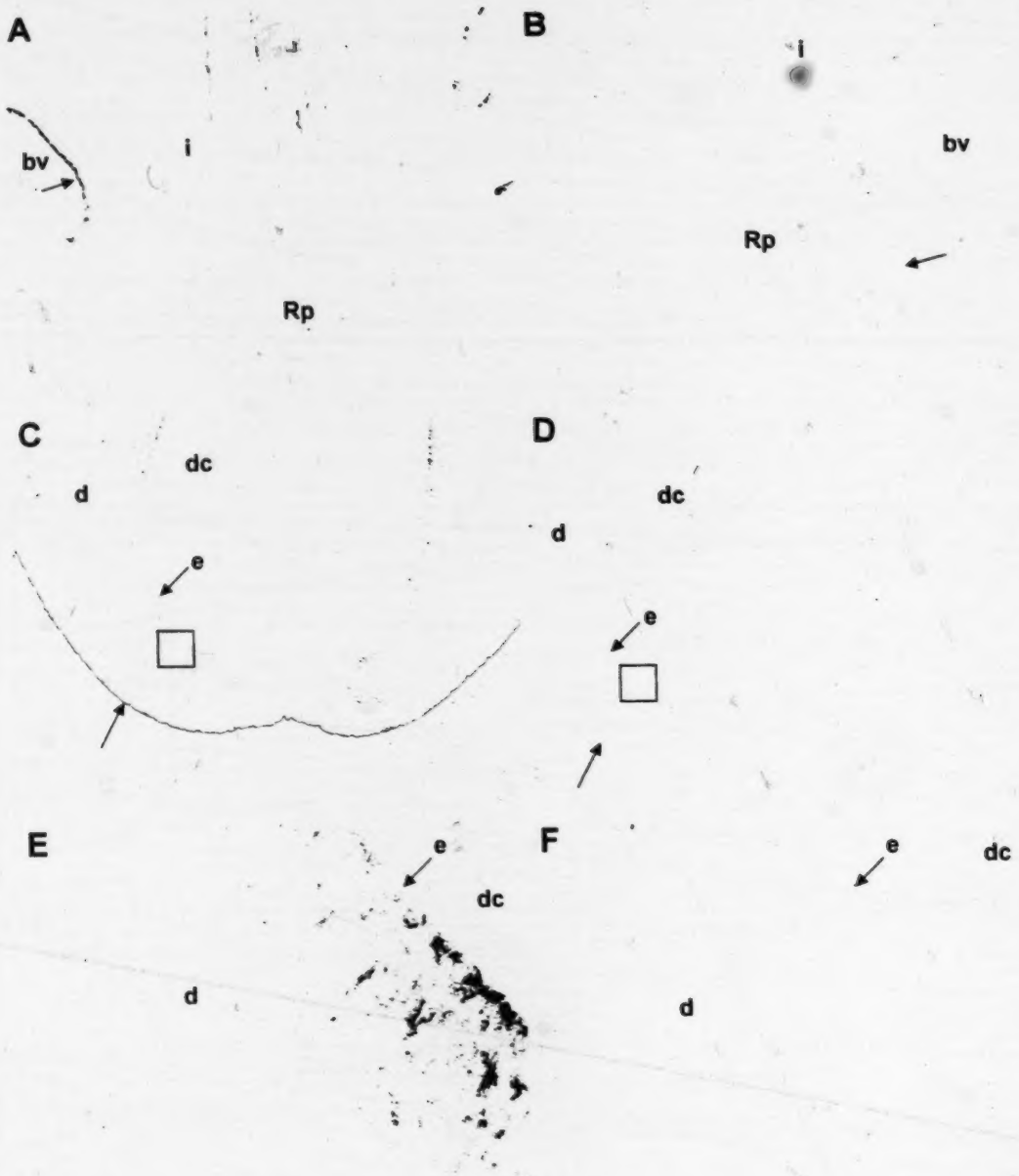
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Fig 3.3 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A) Intense TSH immunoreactivity in the ependymal cells (e) of the spinal cord (sc) and outer epidermis (arrow). Faint TSH staining is also present in the dorsal root ganglia (d) but is not present in marginal (ml) or mantle (m) layers of white matter, nor in the notochord (n). Magnification x100, except in the insert (x1000). (B) Intense Pit-1 immunoreactivity is present in the upper marginal layer (ml) of the spinal cord (sc) and its ependymal lining (e), as well as in the dorsal root ganglia (d) and surrounding myotome (my). Pit-1 immunoreactivity is not present in the mantle (m) layer of the spinal cord, nor in the notochord or the surrounding notochordal sheath. Magnification x100, except in the inset (x1000). (C) Higher magnification (x1000) of the marginal layer (ml) and mantle (m) of the spinal cord. (D) Faint TSH immunostaining in the dorsal root ganglia. Magnification x1000. (E) and (F) Higher magnifications (x1000) of the ependyma and myotome shown in (B). TSH immunoreactivity in (A) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.

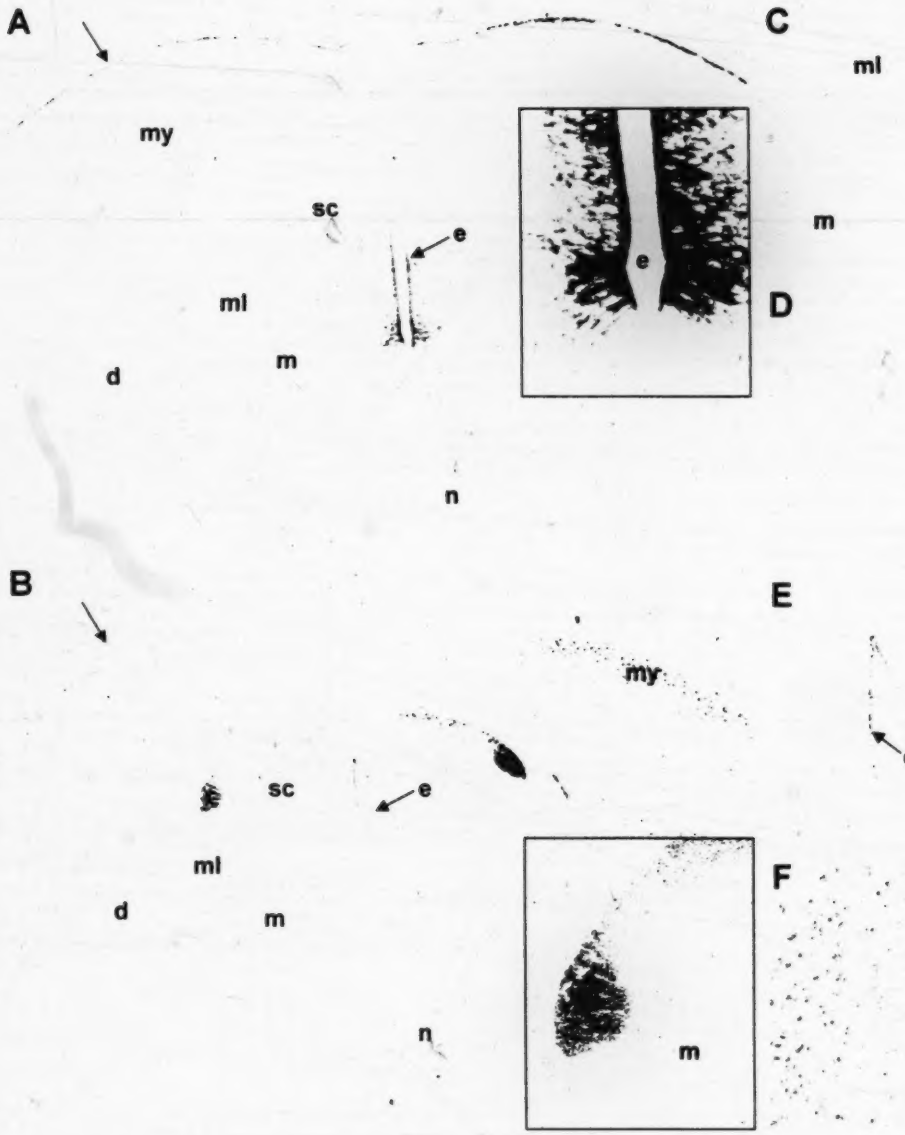
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Fig 3.4 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A) Widespread TSH immunoreactivity in present in most cells of the limb (wing) bud. Labeling is most intense in pockets of the brachial nerve plexus (inset) and in the outer epidermis (arrow), although the dorsal (d) and ventral (v) muscle masses of the wing and chondrocytes in the humerus (h) are also immunostained. Magnification x100. (C) and (D) show magnifications of the insets (x400, x1000, respectively). (B), (E) and (F) Pit-1 immunoreactivity is not present in similar sections of the wing bud.

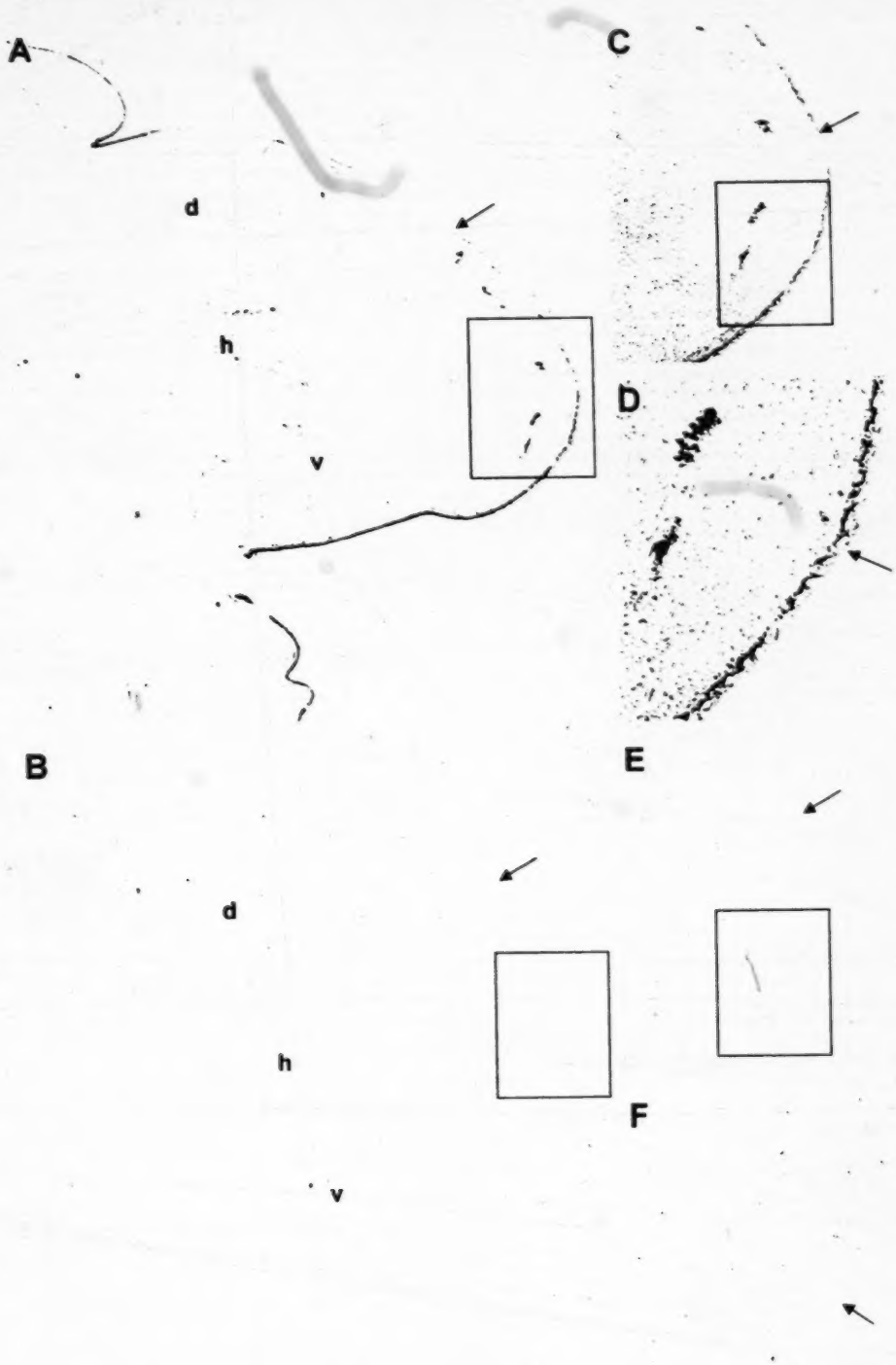
A
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Fig 3.5 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A) Intense TSH immunoreactivity in a developing bronchus in the lung. Magnification x1000. (B) Pit-1 immunoreactivity is not present in a lung bud bronchus. Magnification x1000. (C) Intense TSH immunoreactivity in the proventriculus (crop) of the alimentary canal. Magnification x1000. (D) Pit-1 immunoreactivity in a single layer of adluminal cells in the proventriculus. Magnification x1000. TSH immunoreactivity in (A) and (C) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.

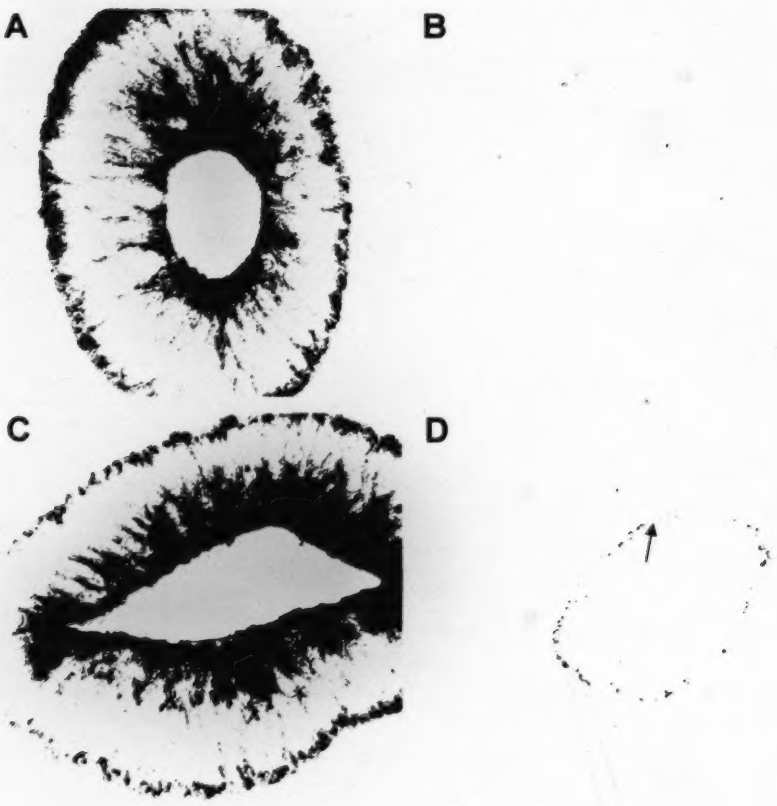
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Fig 3.6 TSH and Pit-1 immunostaining in the trunk of ED 7 chick embryos. (A) Intense TSH immunoreactivity in a single layer (arrow) of cells lining the pleural cavity (pc). Magnification x1000. (B) Pit-1 immunoreactivity is not present in a similar section of the pleural cavity. Magnification x1000. (C) TSH immunolabeling of hepatocytes in the liver. Immunostaining was not present in blood cells (arrow). Magnification x1000. (D) Pit-1 immunoreactivity in liver hepatocytes but not blood cells (arrow). Magnification x1000. TSH immunoreactivity in (A) and (C) is modified from data presented by Murphy and Harvey (2001) and is shown for comparison.

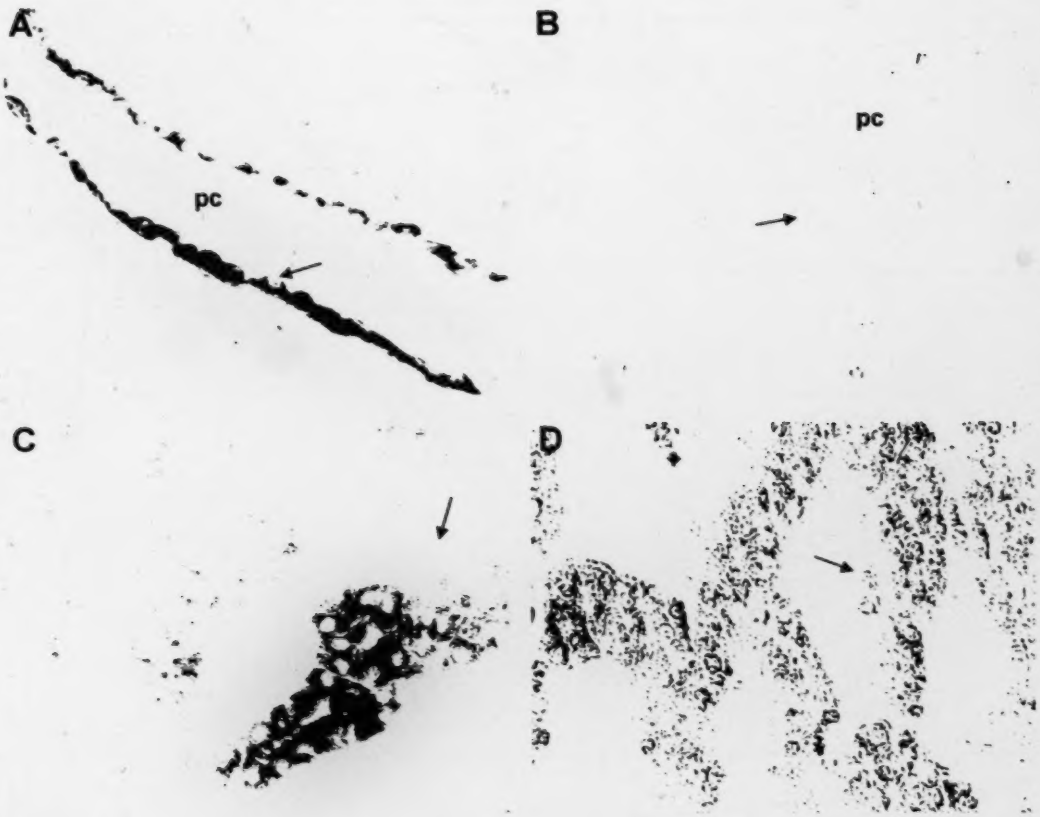

A
pc
B
pc
C
D

Fig 3.7 Western analysis of Pit-1-like proteins in tissues of ED 7 chick embryos. Homogenates of adult chicken pituitary, ED 7 head, trunk, wingbud and whole eye were subjected to Western blotting. Pit-1-like proteins of appropriate size (about 33 kDa) are present in all tissues of the ED 7 chick embryo and correspond to the 33 kDa band seen in the adult chicken pituitary (positive control). Protein homogenates were obtained from a pool of at least 5 embryos.

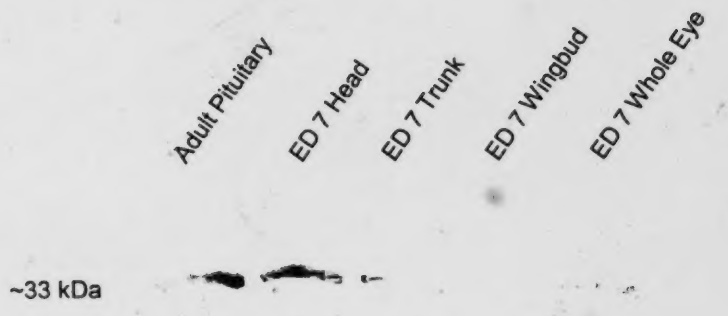
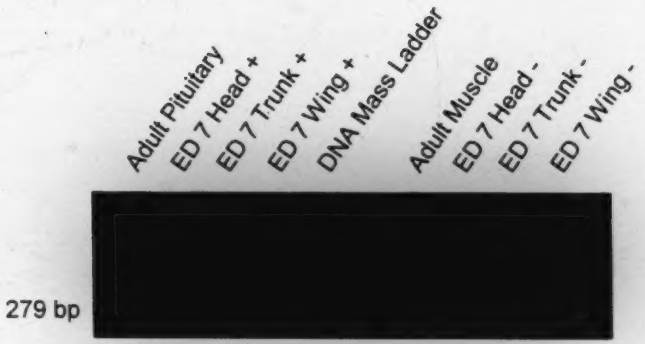
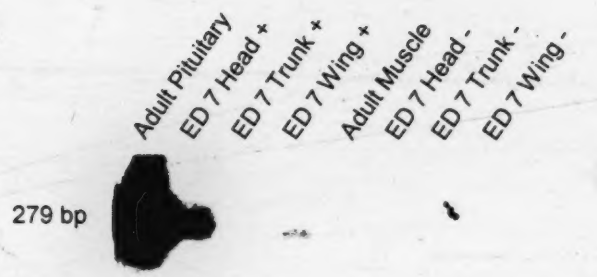

Adult Pituitary
ED 7 Head
ED 7 Trunk
ED 7 Wingbud
ED 7 Whole Eye
~33 kDa

Fig 3.8 RT-PCR and Southern Blotting for Pit-1 in ED 7 chick embryos. (A) RT-PCR of Pit-1 mRNA in ED 7 chick embryos. Total tissue RNA was extracted and reverse transcribed. The cDNA was then amplified in the presence of oligonucleotide primers (t11 and t12) designed to amplify a 279 bp fragment of chicken pituitary Pit-1 cDNA (Harvey *et al* 2000). This fragment was amplified from reverse transcribed RNA (+) extracted from the ED 7 head, trunk and wingbud and adult chicken pituitary (positive control), but was only visible in the pituitary with ethidium bromide staining. This fragment was not evident when PCR was performed with RNA in the absence of reverse transcriptase (-) or in the adult chicken muscle (negative control). (B) Southern blotting of PCR products amplified in the presence of oligonucleotide primers designed to amplify a 279 bp fragment of chicken pituitary Pit-1 cDNA, using a DIG-labeled Pit-1 RNA probe. RNA from ED 7 head, trunk and wingbud produced a 279 bp Pit-1 cDNA after RT-PCR and Southern blotting (+), but not in the absence of reverse transcriptase (-).

A


Adult Pituitary
ED 7 Head +
ED 7 Trunk +
ED 7 Wing +
DNA Mass Ladder
Adult Muscle
ED 7 Head -
ED 7 Trunk -
ED 7 Wing -
279 bp

B


Adult Pituitary
ED 7 Head +
ED 7 Trunk +
ED 7 Wing +
Adult Muscle
ED 7 Head -
ED 7 Trunk -
ED 7 Wing -
279 bp

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Chapter 4

Extrapituitary Growth Hormone: Under-estimation of Immunohistochemical Staining by Carnoy's Fixation

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A version of this paper has been submitted to the *Journal of Endocrinology* for publication. Murphy AE, Peek H, Harvey S. Extrapituitary growth hormone: under-estimation of immunohistochemical staining by Carnoy's fixation. The author would like to thank Hilke Peek for laboratory assistance.

I. INTRODUCTION

Growth hormone (GH) gene expression primarily occurs in pituitary somatotrophs, although numerous extrapituitary sites of GH expression have been documented. Indeed, in domestic fowl, GH mRNA has been identified in the brain (Render *et al.*, 1995a), in the thymus, bursa and spleen (Render *et al.*, 1995b), in the testes and vas deferens (Harvey *et al.*, 2002; Murphy *et al.*, 2002), in the ovary (Luna *et al.*, 2000), in the heart (Takeuchi *et al.*, 2001) and in the eye (Takeuchi *et al.*, 2001). In mammalian species, GH gene expression has additionally been determined in the placenta (Schwarler *et al.*, 1997), mammary gland (Mol *et* al., 1995a, b), liver (Recher *et* al., 2001), lung (Allen *et* al., 2000), salivary gland (Tresguerres *et al.*, 1999), skin (Palmetshofer *et al.*, 1995; Slominski *et al.*, 2000) and in smooth muscle and endothelial cells of blood vessels (Wu *et al.*, 1996; Recher *et al.*, 2001). Proteins with GH-immunoreactivity are also present in these sites and in other peripheral tissues (eg. in teeth, kidney and gut; Kyle *et al.*, 1981; Costa *et al.*, 1993; Zhang *et al.*, 1997). Nevertheless, with the exception of limited studies on the nervous (Lechan *et al.*, 1981; Ramesh *et al.*, 2000, Takeuchi *et al.*, 2001) and immune (Kao *et al.*, 1992; Maggiano *et al.*, 1994) systems, this immunoreactivity has been determined by radioimmunoassay of tissue extracts and its cellular location is largely unknown. Two immunohistochemical studies on the localization of GH in peripheral tissues of embryonic chicks have, however, been published (Harvey *et al.*, 2000; Murphy & Harvey, 2001).

In one study, GH-immunoreactivity was found to be abundant and widespread in the bodies of embryonic tissues. This immunoreactivity was thought to be specific, since it was detectable by three different GH antibodies (two polyclonals, one monoclonal) and

was completely lost following pretreatment of the primary antibodies with excess recombinant GH. A different tissue distribution of GH immunoreactivity was, however, observed in a more recent study (Murphy & Harvey, 2001), in which the cellular distribution of GH was restricted to discrete cells or tissue layers. The main difference between these two studies was the fixative used for immunohistochemistry: 4% paraformaldehyde (Harvey *et al.*, 2000) and Carnoy's fixative (Murphy & Harvey, 2001). Since different fixatives can result in quantitative and qualitative changes in protein detection (Chiu *et al.*, 1994; Vince *et al.*, 1997; Hemmer *et al.*, 1998; Giaccone *et al.*, 2000), the influence of fixation on the immunocytochemical detection of GH in chick embryos was therefore empirically assessed.

II. MATERIALS AND METHODS

Tissues

Fertile White Leghorn chicken eggs from the University of Alberta Poultry Unit were incubated at 37 °C in humidified air. The eggs were turned one quarter of a revolution each day during the incubation period. Whole embryos at embryonic day 7 (ED 7) of the 21 day period of embryogenesis were dissected and decapitated in phosphate-buffered saline (PBS, pH 7.4) prior to fixation. ED 7 embryos were used since pituitary somatotrophs do not differentiate until ED 12 to ED 14 (Porter, 1997) and hence tissue GH-immunoreactivity could not be due to the sequestration of pituitary GH. Peripheral tissues were similarly used to ensure that GH-immunoreactivity was not due to pituitary GH detection.

Fixation

Tissues were collected into freshly prepared paraformaldehyde (4% w/v, in PBS) (Sigma, Mississauga, Ontario, Canada) or Carnoy's fluid (60% ethanol v/v; 30% chloroform v/v; 10% acetic acid v/v). Paraformaldehyde is a cross-linking fixative that forms links (hydroxyl-methylene bridges) between reactive end-groups of adjacent protein chains, whereas Carnoy's is a precipitant fixative that denatures proteins by destroying the hydrophobic bonds that hold together the tertiary structures of large protein molecules.

Immunohistochemistry

The headless embryos were fixed, overnight, at 4 °C. They were then dehydrated in a graded series of alcohol (50% 15 min; 70% 15 min; 95% 30 min; 100% 2x30 min) and cleared with Hemo-de (Fisher Scientific, Edmonton, Alberta, Canada) for 2x30 min. The tissues were then infiltrated with paraffin wax to 24 h at 60 °C under normal atmospheric pressure. Serial transverse (8 μm) sections were then taken using a microtome and mounted into charged slides (Fisher Scientific). Immunocytochemical staining was performed with commercial reagents (Vector Laboratories, Burlingame, CA, USA; Sigma) using the avidin-biotin-peroxidase (ABC) method (Hsu *et al.*, 1981). Sections were incubated overnight at 4 °C with a specific polyclonal antisera raised in rabbits against native (pituitary) chicken GH (Harvey *et al.*, 2000), diluted 1:1000 in 1% normal goat serum. After incubation, the slides were washed three times for 5 min in PBS. Sections were then incubated for 1 h at room temperature in biotinylated goat anti-rabbit immunoglobin G (IgG) (Sigma; 1:500). The slides were then washed in PBS and

incubated in ABC reagent for 1 h at room temperature and washed in PBS. Staining was visualized using the chromogenic substrate, diaminobenzidine tetrahydrochloride (DAB) (Sigma), which resulted in a brown coloration. The specificity of staining was determined by preabsorbing the GH antisera with recombinant chicken GH (Amgen, Thousand Oaks, CA, USA; 1 mg/ml) for 1 h prior to section incubation. All tissues collected in paraformaldehyde or Carnoy's fluid were processed together under identical conditions with the same reagents. Digital images were collected using a SPOT digital Microscope Camera (Carsen Group, Markham, Ontario, Canada) mounted on an Olympus B microscope.

III. RESULTS

After Carnoy's fixation, intense immunocytochemical staining for GH was seen in the spinal cord, although restricted to the marginal cells of the white matter, to the ventral horn and mantle, to spinal nerves, and to the ventral root nuclei (Fig. 4.1A, B, C). The surrounding tissue was, however, only faintly stained or unstained. In contrast, strong staining was seen throughout the spinal cord following paraformaldehyde fixation, including the ependymal cells surrounding the spinal canal (Fig. 4.1C, D). In contrast to the tissues fixed in Carnoy's, the tissues fixed in paraformaldehyde had intense GH-immunoreactivity in both the ventral and dorsal root ganglia (Fig. 4.1C, D). Similarly, while GH-immunoreactivity was abundant and widespread in the notochord, notochordal sheath and in the myotome of paraformaldehyde-fixed tissues (Fig. 4.2B, D), GH-immunoreactivity was not detectable in these tissues following Carnoy's fixation (Fig. 4.2A, C).

A single layer of epithelial cells lining the esophagus (crop) had GH-immunoreactivity following Carnoy's fixation (Fig. 4.3A), although intense GH-immunoreactivity was seen in numerous cells in the mucosal epithelium, submucosa and muscular layers of this structure following paraformaldehyde fixation (Fig. 4.3B). Strong GH staining was, likewise, seen in the bronchus after fixation in paraformaldehyde (Fig. 4.3D), but only faint staining was seen after fixation in Carnoy's (Fig. 4.3C). Similar differences in GH staining were also seen in the heart, in which intense staining was present in the myocardium of the atria and in atrial cushions (Fig. 4.4B) and ventricles (Fig. 4.5C, D) fixed in paraformaldehyde, but not in the atria (Fig. 4.4A) or ventricles (Fig. 4.5A, B) fixed in Carnoy's. Strong GH-immunoreactivity was also seen in the cells lining the pericardial cavity and in the body wall of embryos fixed in paraformaldehyde (Fig. 4.4C, D), but not in embryos fixed in Carnoy's (Fig. 4.4A, B). Although intense staining was present in the myocardial cells of the heart following paraformaldehyde fixation, only faint, diffuse staining was present in blood cells in the atria and ventricles and in the pericardial space (Fig. 4.4C, D, Fig. 4.5C, D). The atrio-pulmonary septum surrounding the ventricles was also only slightly stained following paraformaldehyde fixation (Fig. 4.5C, D). In marked contrast, strong and discrete GH staining was localized in the extensor nerve of the anterior limb bud fixed in Carnoy's (Fig. 4.6A), whereas GH-immunoreactivity was widespread in the limb bud fixed in paraformaldehyde. Following paraformaldehyde fixation, GH-immunoreactivity was particularly abundant in chondrocytes of the humerus (Fig. 4.6F) (as in chondrocytes in the ribs, Fig. 4.1D) and in a layer of outer epidermal cells (Fig. 4.6E), which were faintly stained or unstained after Carnoy's fixation (Fig. 4.6B, C). Intense GH-immunoreactivity is present in the dorsal

and ventral muscle mass of the wing bud following paraformaldehyde fixation (Fig. 4.6D), but not after Carnoy's (Fig. 4.6A).

In all tissues fixed in Carnoy's or paraformaldehyde, the GH staining was specific and completely lost following preabsorption of the primary antibody with excess recombinant GH (data not shown).

IV. DISCUSSION

These results clearly show the presence of GH-like proteins in peripheral tissues of early chick embryos, but demonstrate tissue-specific differences in staining following paraformaldehyde or Carnoy's fixation.

The distribution of GH-immunoreactivity in the peripheral tissues of paraformaldehyde-fixed ED 7 chicks was widespread, but particularly striking in the spinal cord, dorsal and ventral root ganglia, notochord, myotome, epidermis, heart and the humerus. These findings extend preliminary observations by Harvey *et al.* (2000) using paraformaldehyde-fixed tissues from ED 6 and ED 7 embryos. The cellular localization of GH staining in the neural tube, dorsal and ventral root ganglia, notochord, heart and limb bud is, however, much clearer in this study and presented at high magnification for the first time. The staining for GH in these tissues is clearly cellular and within the cytoplasm, whereas it could not be clearly differentiated from background staining in the earlier study (Harvey *et al.*, 2000). The presence of GH staining in the esophagus (crop), lung (bronchus), myotome and the anterior extensor nerve following paraformaldehyde staining are novel observations.

A widespread distribution of GH-immunoreactivity might be expected, since the headless bodies of ED 6 and ED 8 embryos readily express the GH gene (Harvey *et al.*, 2000). A widespread cellular distribution of GH-immunoreactivity might also be expected in view of the finding, in mammals, of GH mRNA and immunoassayable GH in extracts of most peripheral tissues (eg. Kyle *et al.*, 1981; Costa *et al.*, 1993; Wu *et al.*, 1996; Recher *et al.*, 2001), particularly in perinatal animals (Costa *et al.*, 1993; Recher *et al.*, 2001). A widespread distribution of GH-immunoreactivity might also be expected, since GH may act as an autocrine or paracrine growth factor during embryonic development (Harvey & Hull, 1997) and GH receptors (Harvey *et al.*, 2000) and a GH responsive gene (Harvey *et al.*, 2001a) are ubiquitous in embryonic tissues.

The distribution of GH-immunoreactivity in ED 7 tissues fixed in Carnoy's was noticeably different from that observed following paraformaldehyde fixation. After Carnoy's, the immunohistochemical staining of GH was more discrete (mainly restricted to marginal and mantle layers of the spinal cord, the ventral root ganglia, to spinal nerves and to the anterior extensor nerve). These results are similar to those published by Murphy and Harvey (2001), in which GH staining was also observed in the trigeminal nerve and vagal nerve following Carnoy's fixation. This suggests that Carnoy's fixative preferentially detects GH-immunoreactivity in neural tissues. The discrete presence of GH in the extensor nerve is a novel finding of this study, as is the absence of GH staining in chondrocytes and cardiac cells fixed in Carnoy's fluid. Indeed, apart from the neural derivatives, GH-immunoreactivity is barely detectable in the peripheral tissues of embryos fixed in Carnoy's. It is therefore of interest that the GH staining in the heads of ED 7 chicks fixed in Carnoy's (Murphy & Harvey, 2001) was also much less than in

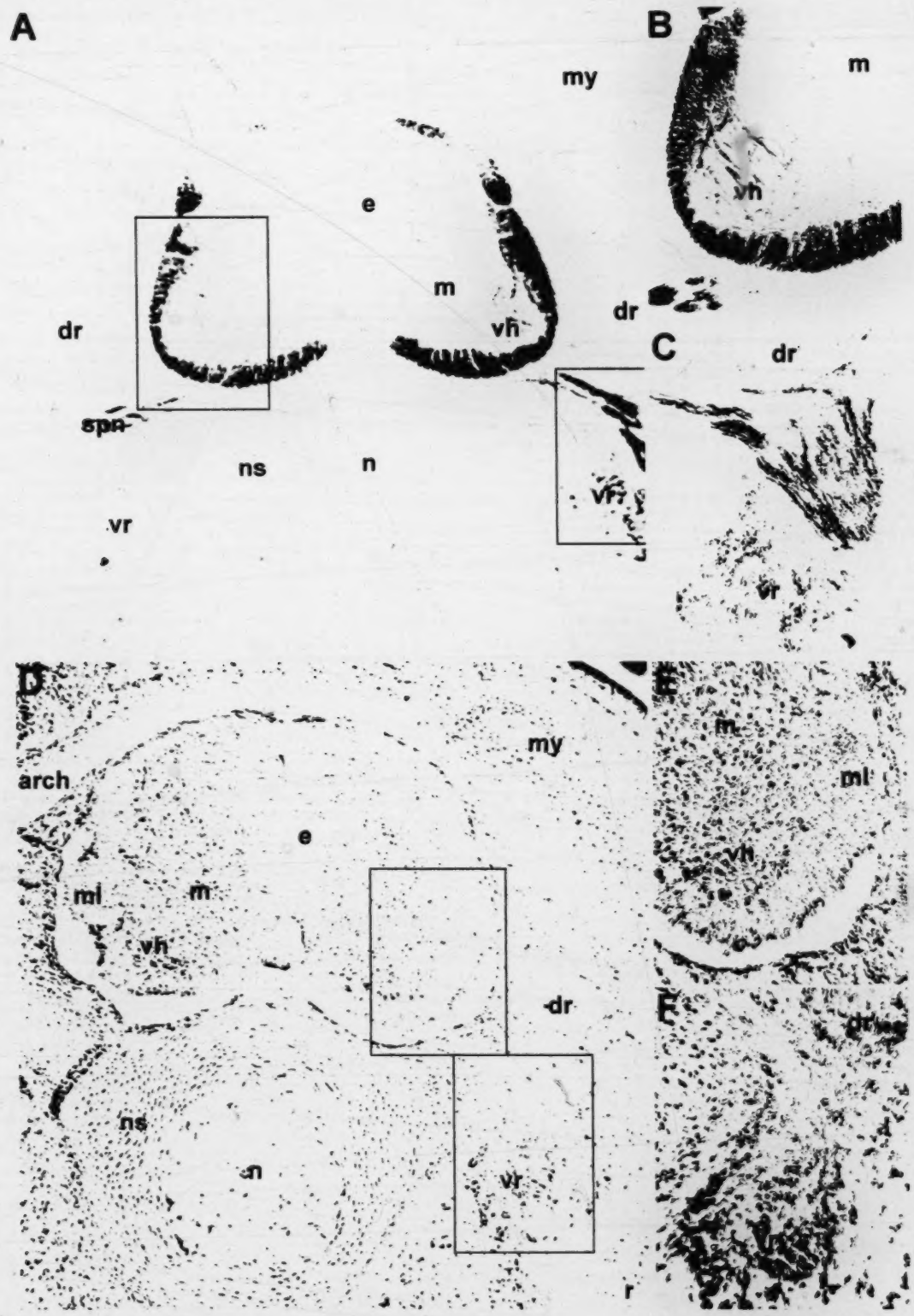
heads fixed in paraformaldehyde (Harvey *et al.*, 2001b). This is particularly evident for Rathke's pouch (a non-neural pharyngeal derivative that differentiates into the pituitary gland), which is almost devoid of GH-immunoreactivity following Carnoy's fixation (Murphy & Harvey, 2001) but abundantly stained for GH-immunoreactivity following paraformaldehyde fixation (Harvey *et al.*, 2001b). The controversy in the literature (Porter, 1997) on the ontogeny of pituitary somatotrophs (ED 4.5, Thommes *et al.*, 1987 (Bouin's fixation); ED 10, Malamed *et al.*, 1993 (Zenker's fixative); ED 12, Jozsa *et al.*, 1979; Allaerts *et al.*, 1999 (Zamboni's fluid); ED 12, Porter *et al.*, 1995 (B-5: formalin-mercuric chloride)), might therefore partly reflect differences in the fixatives used. Qualitative differences in the distribution of GH staining in other tissues of embryonic chicks may similarly reflect differences in tissue fixation. For instance, while Harvey *et al.* (2001b) observed widespread staining for GH in neural retinas fixed in paraformaldehyde, Takeuchi *et al.* (2001) found GH-immunoreactivity only in pigmented retinal epithelial cells following fixation in Bouin's (a picric acid-based fixative).

Paraformaldehyde is a cross-linking fixative and is particularly useful for fixing smaller proteins and peptides (Polak & Van Noorden, 1997). Carnoy's, in contrast, is a precipitant fixative and more useful for the fixation of large proteins. Carnoy's fluid is a better fixative than paraformaldehyde in preserving tissue architecture and in facilitating tissue sectioning (Polak & Van Noorden, 1997), but it reduces specific staining for GH-immunoreactivity. Carnoy's fixative has similarly been found to reduce antigen staining in other immunocytochemical studies, in comparison with formalin or paraformaldehyde (e.g. Cammer *et al.*, 1985; Schutte *et al.*, 1987; Bos *et al.*, 2000). It is therefore possible that Carnoy's and paraformaldehyde fixatives may differentially fix the different GH

moieties present in neural and non-neural tissues. It is now well established that pituitary GH comprises a family of size and charge variants that may result from differential gene transcription or post-translational modifications (Aramburo *et al.*, 2000, 2001a, b; Martinez-Coria *et al.*, 2002). This may include oligomerization (into dimmers, trimers, quadramers and pentamers) and proteolytic cleavage (into fragments of 15 kDa and 7 kDa) of the monomer (26 kDa) moiety (Aramburo *et al.*, 2001b). Small GH moieties of 14 kDa to 17 kDa have also been identified in pituitary extracts and, interestingly, these are preferentially produced in embryonic chicks (Aramburo *et al.*, 2000). GH size heterogeneity has also been identified in the testes of embryonic and adult chickens (Luna *et al.*, 2000, 2002), in which the submonomer variants are the most abundant moieties, particularly in embryonic tissues. Submonomer GH variants are also present in the eyes of chick embryos, but not in the eyes of neonatal chicks (Takeuchi *et al.*, 2001). Submonomer GH moieties, therefore, appear to be more abundant in peripheral extrapituitary tissues than in the pituitary and more abundant in embryos than in neonatal or adult chickens. It is, therefore, possible that these smaller moieties are not fixed by Carnoy's fluid, under-estimating the GH-immunoreactivity that is detected by the cross-linking of small and large GH proteins during paraformaldehyde fixation. Fixation with Carnoy's fluid does, however, appear to selectively label GH in neural derivatives, and it may be more useful than paraformaldehyde for immunohistochemical studies on neural GH. This selectivity may, interestingly, reflect the fact that no submonomer GH moieties are present in Western blots of hypothalamic and extrahypothalamic brain tissues (Render *et al.*, 1995a), whereas submonomer GH moieties are abundantly present in the other non-neural tissues (Render *et al.*, 1995a; Luna *et al.*, 2000).

In summary, qualitative and quantitative differences in tissue GH-immunoreactivity are evident in immunohistochemical studies employing paraformaldehyde and Carnoy's fixatives. The presence of GH-immunoreactivity in the embryonic chick is under-estimated by fixation in Carnoy's fluid, and caution should be used in interpreting immunohistochemical data obtained with this fixative.

Fig 4.1 GH –immunoreactivity in the spinal cord of ED 7 chick embryos. Following fixation in Carnoy's fluid (A, B, C), intense staining is present in the marginal layer (ml), spinal nerves (spn) and the ventral root nuclei (vr). Light staining is also present in the ventral horn (vh) and the mantle region (m). Magnification: A x100; B x400; C x400. Following fixation in 4% (w/v) paraformaldehyde, GH staining is present through the spinal cord, including the ependymal cells (e) surrounding the spinal canal and the dorsal root nuclei (dr) (D, E, F). GH-immunoreactivity is also present in the ventral root nuclei (vr) and the surrounding myotome (my), vertebral arch (arch), notochord (n), notochordal sheath (ns) and the head of a rib (r). Magnification: D x100; E x400; F x400.


A
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vr
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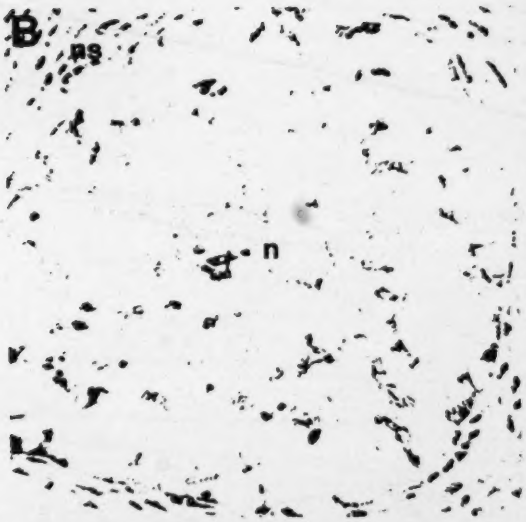
ml

Fig 4.2 GH-immunoreactivity in the notochord and myotome of ED 7 chick embryos. Growth hormone (GH) –immunoreactivity in the notochord (n), notochordal sheath (ns; B) and myotome (my; D) of embryonic chicks following fixation in 4% (w/v) para-formaldehyde, in comparison with comparable tissues fixed in Carnoy's fluid (A, C). Magnification x400.

A

ns

n



C

my

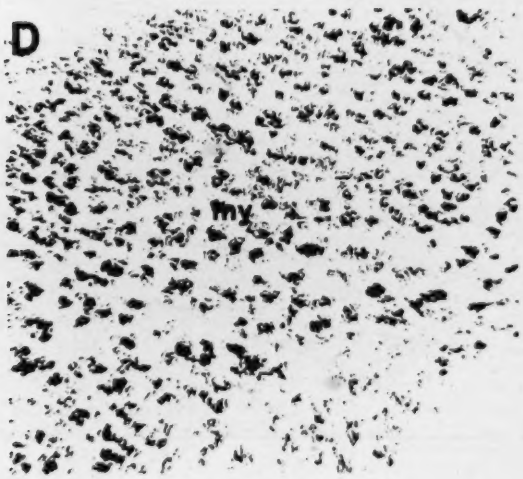
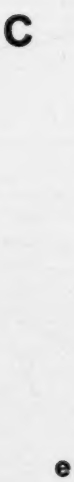


Fig 4.3 GH-immunoreactivity in the esophagus and bronchus of ED 7 chick embryos. GH –immunoreactivity in the esophagus (B) and bronchus (D) of embryonic chicks following fixation in 4% (w/v) paraformaldehyde, in comparison with tissues fixed in Carnoy's fluid (A, C): Abbreviations: e, epithelium; s, submucosa; m, esophageal muscularis. Magnification x400. Only a single layer of esophageal epithelial cells (arrowed) was GH-immunoreactive following Carnoy's fixation, whereas GH-immunoreactivity was widespread following paraformaldehyde fixation.

A
m
s
e

B
s
e


C
e

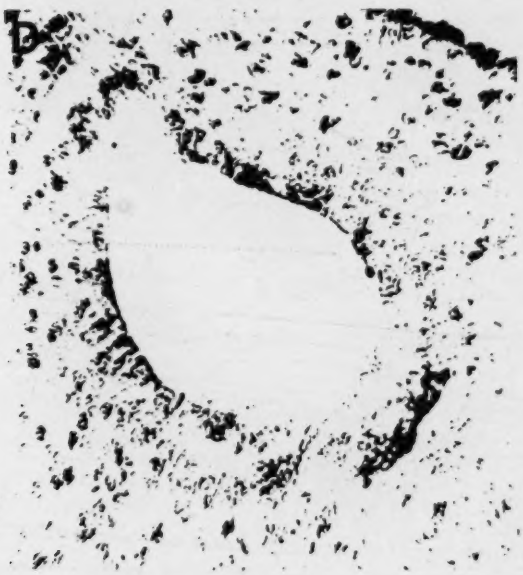


Fig 4.4 GH-immunoreactivity in the heart atria of ED 7 chick embryos. GH – immunoreactivity in the atria of embryonic chicks following fixation in 4% (w/v) paraformaldehyde (C, D), in comparison with atria fixed in Carnoy's fluid (A, B). Intense GH-immunoreactivity is present throughout the myocardium (mc) of the right atria (ra), atrial cushion (ac) and the body wall (bw) following fixation in paraformaldehyde (C, D). Only faint, diffuse staining to blood cells (arrowed) is seen in the atrium and pericardial cavity. Magnification: A, C x100; B, D x400. Abbreviation: avc, atrio-ventricular canal.

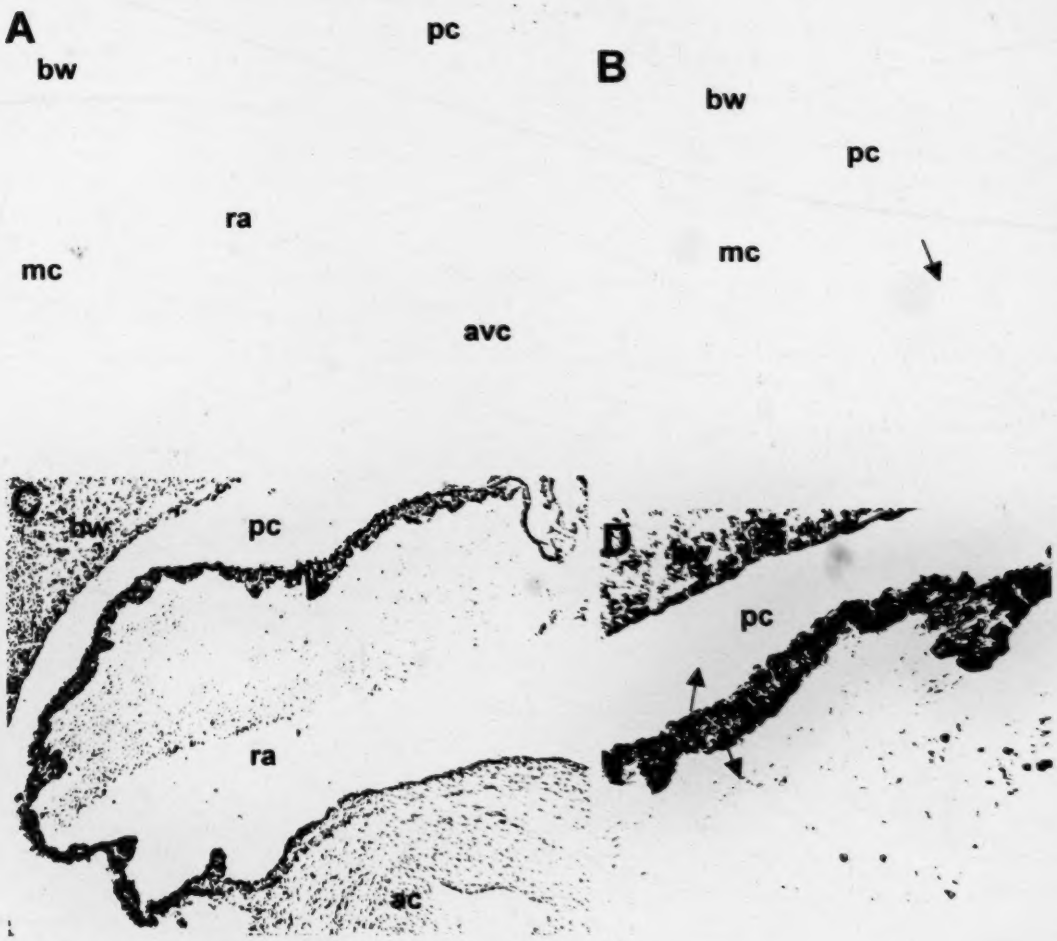

A
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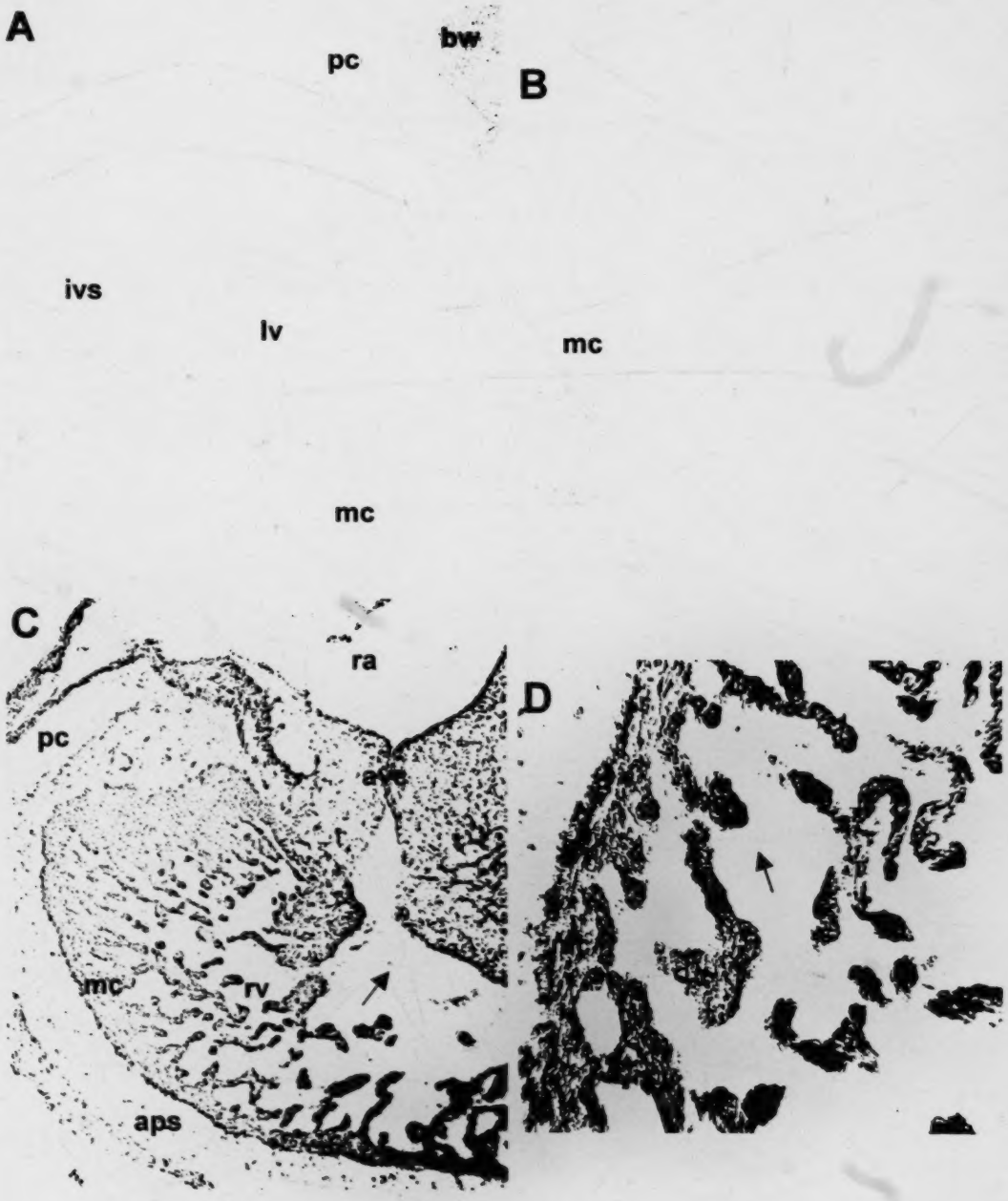
Fig 4.5 GH-immunoreactivity in the heart ventricles of ED 7 chick embryos. GH – immunoreactivity in the ventricles of chick embryos following fixation in 4% (w/v) paraformaldehyde (C, D), in comparison with ventricles fixed in Carnoy's fluid (A, B). Intense GH-immunoreactivity is present throughout the ventricular myocardium (mc) of tissues fixed in paraformaldehyde, whereas only faint GH staining is present in the aortico-pulmonary septum (aps) or to blood cells (arrowed) in the ventricular lumen. In contrast, GH-immunoreactivity is not seen in ventricular tissue fixed in Carnoy's (A, B). Abbreviations: ra, right atrium; avc, atrio-ventricular canal; ivs, intraventricular septum; bw, body wall; pc, pericardial cavity. Magnification: A, C x100; B, D x400.


A
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bw
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ivs
lv
mc
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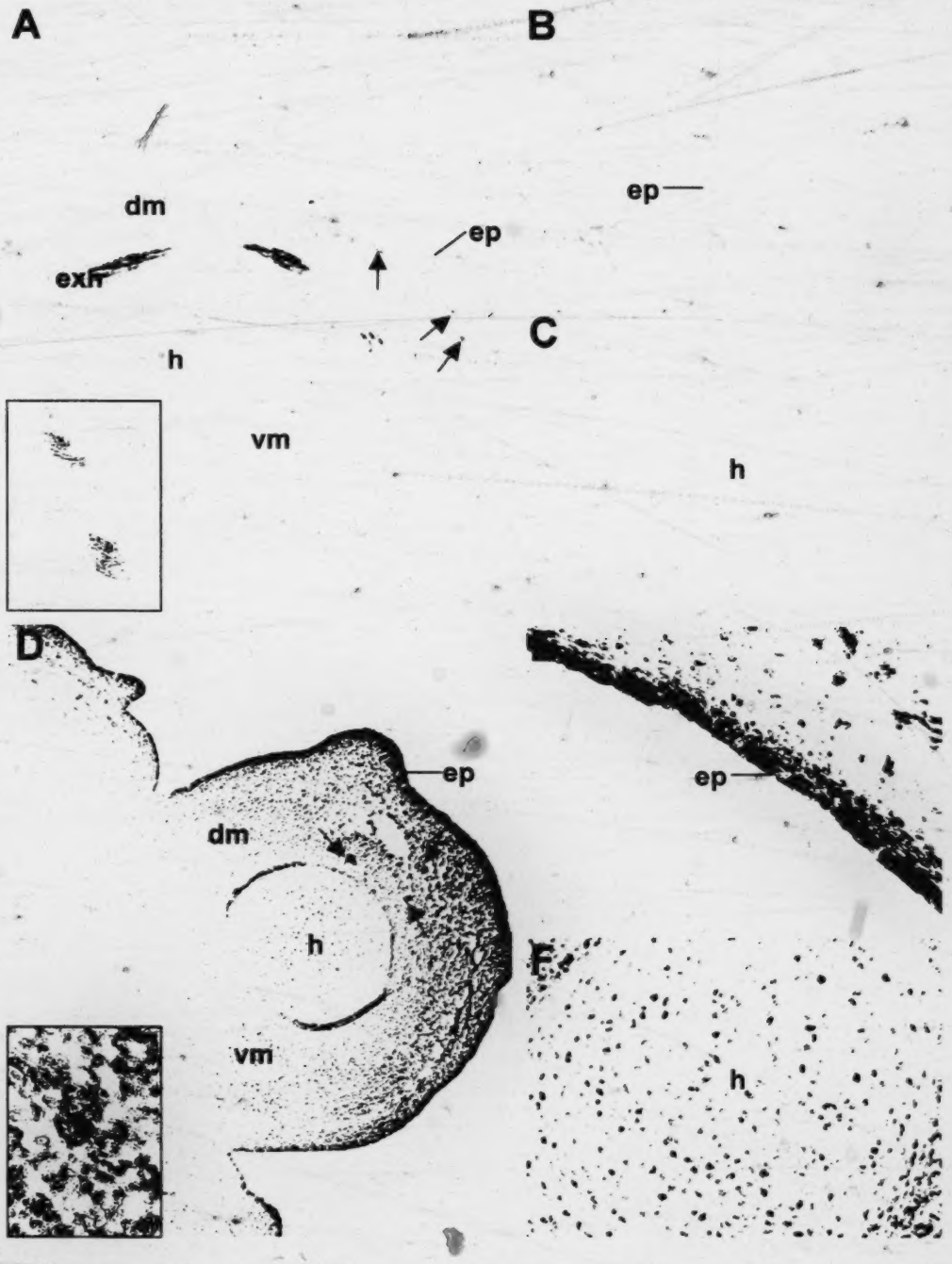
C
ra
pc
mc
rv
aps
D

Fig 4.6 GH-immunoreactivity in the wing bud of ED 7 chick embryos. GH – immunoreactivity in the chick embryo wingbud following fixation in Carnoy's fluid (A, B, C) or 4% (w/v) paraformaldehyde (D, E, F). Intense GH staining is present in the extensor nerve (exn, and arrows) following fixation in Carnoy's (A, B), although cells in the dorsal (dm) and ventral (vm) muscle masses, the epidermis (ep) and the humerus (h) were unstained. In contrast, strong GH-immunoreactivity is seen throughout the wingbud following paraformaldehyde fixation, but is particularly striking in the extensor nerve (arrows), muscle masses, epidermis and humerus (D, E, F). Magnification: A, D x100; B, C, E, F x400. The insets show tracts of extensor nerve at x1000 magnification.


A
B
dm
ep
exn
ep
C
h
vm
h
D
ep
ep
dm
h
vm
E
h

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Chapter 5

Growth Hormone (GH) and GH-receptor mRNA in the Male Reproductive Tract: Evidence for Autocrine/Paracrine Roles of GH in Avian Reproduction

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I. INTRODUCTION

Growth hormone (GH) is primarily produced in the pituitary gland, although it is well established that some extrapituitary tissues express the GH gene (Harvey and Hull, 1997). Amongst these, the testis is thought to be a site of GH synthesis, since GH gene transcripts are present in the human testis (Untergasser *et al.*, 1996; 1997; 1998; Berger *et al.*, 1999). However, while the pituitary (GH-N) gene is transcribed in the human testis, 'placental' GH variants (GH-V) are preferentially expressed (Untergasser *et al.*, 1997; 1998; Berger *et al.*, 1999). The possibility that GH gene expression occurs in the testis of other species has yet to be determined, although proteins with GH-immunoreactivity have been demonstrated in the testes of fetal mice (Nguyen *et al.*, 1996) and the Wolffian ducts of embryonic chickens (Wang, 1989; Harvey *et al.*, 2000a). The possible expression of the GH gene in the male reproductive tract was therefore examined in the domestic fowl, in which GH mRNA has previously been identified in the brain (Render *et al.*, 1995a), spleen, thymus and bursa (Render *et al.*, 1995b), heart and eye (Takeuchi *et al.*, 2001). In addition, as roles for GH in male reproduction have been described (Hull and Harvey, 2000b), the possible presence of GH-receptor (GHR) mRNA in the male reproductive tract was also assessed.

II. MATERIALS AND METHODS

Adult male White Leghorns from the University of Alberta Poultry Station (Edmonton) were killed by cervical dislocation and the testes, upper vas deferens (1 cm adjacent to the testis), the middle vas deferens and the cloaca were rapidly collected and frozen in liquid nitrogen. The liver and pituitary glands were collected from some birds

as negative and positive controls for the presence of GH mRNA (Render *et al.*, 1995a) and as positive controls for the presence of GHR mRNA (Hull *et al.*, 1995).

RT-PCR

The presence of GH and GHR mRNA in reproductive tissues was assessed by RT-PCR. Total cellular RNA was extracted using commercial reagents (Trizol, Gibco, BRL, Burlington, Ontario, Canada) and analyzed for purity and content, respectively, by spectrophotometry at 260 nm and electrophoresis in ethidium-bromide-stained 1.5% (w/v) agarose minigels (Render *et al.*, 1995a; 1995b). RNA samples (1 μg) were reverse-transcribed using superscript (100 U; BRL) in the presence of an oligo dt primer and excess deoxynucleotides (Gibco-BRL) during a 1 h incubation at 37 °C and a 30 min incubation at 42 °C. cDNA aliquots (1 μl) were then amplified for 30 cycles in a Thermal Cycler (Fisher Scientific, Edmonton) in a mixture containing 10x PCR buffer (80 mmol KCl/l ; 16 mmol Tris-HCl/l, pH 8.4, 1.5 mmol Mg Cl_2/l; 0.1% (v/v) Triton X-100), deoxynucleotides (200 μm of each), 25 pmol oligonucleotide primers and 5 U Taq DNA polymerase (Boehringer Mannheim). Amplifications were achieved by cycles of denaturation (94 °C for 1 min), annealing (50 °C for 1 min) and extension (72 °C for 2 min), followed by a final period of extension (72 °C for 10 min). The PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels and were visualized by ethidium bromide staining.

Amplifications were in the presence of oligonucleotide primers CLR3 (5^{l}-CGTTCAAGCAACACCTGAGCAACTCTCCCG-3^{l}) and CLR5 (5^{l}-GCCTCAGATGGTGCAGTTGCTCTCTCCGAA-3^{l}), designed to amplify a 689 bp

fragment of the pituitary GH cDNA (Render *et al.*, 1995a), or in the presence of primers khu9 (5'-CCTCGATTTGGATACCATATTGTGTTAAGC-3') and khu10 (5'-CTGTTACGGCCAGCCCACACACTCCGAAG-3') designed to generate a 500 bp fragment of the chicken GHR cDNA coding for the extracellular domain of the chicken GHR (Hull *et al.*, 1995) or in the presence of primers khu11 (5'-CTGCGGCCGCAGGACCAGTCCAAAGATTAA-3') and khu12 (5'-AAGCGGCCGCGGCAGTAGTGGTAAGGCTTTC-3') designed to generate a 800 bp fragment of the chicken GHR cDNA coding for the intracellular domain of the GHR (Hull *et al.*, 1995). The possible presence of Pit-1 mRNA was similarly determined using oligonucleotide primers t15 (5'-GCACTCGTGGTGC TCC TTGAT AATAGA -3') and t14 (5'-ATCCTCATGCG TTTTCTTACCAGTCCCG -3'), designed to generate a 975 bp fragment of chicken Pit-1 cDNA (Harvey *et al.*, 2000b). As a control, the presence of a housekeeping gene (Gap DH) in the RNA extracts was also assessed, using oligonucleotide primers based on the chicken Gap DH sequence: cGAP 5 (5'-CTGGTGTCTTCACCACCATG-3') and cGAP3 (5'-CAGCAGCCTTCACTA CCCTC-3') (Stone *et al.*, 1985).

cDNA Cloning and Sequencing

PCR products were purified using the High Pure™ PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN, USA) and then ligated into pCR®II-TOPO® vectors (Invitrogen Life Technologies Carlsbad, CA, USA). The ligated vector was then transformed into TOP10F' One Shot® chemically competent *E. coli* cells (Invitrogen Life Technologies Carlsbad, CA, USA). The cells were then plated onto Luria-Bertani (LB)

medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0) agar plates containing 50 µg/ml ampicillin and 40 µl X-gal (400 mg X-gal in 10 ml dimethylformamide) for blue/white screening and incubated at 37 °C overnight. The next day, 3-4 white colonies were selected, placed into 5 ml LB medium and incubated at 37 °C with vigorous shaking for 8 h. The cell cultures were centrifuged, excess medium poured off and the wet pellet of cells was purified with a QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, ON, Canada) to isolate plasmid DNA. The inserts were isolated by digestion with *EcoRI* (Gibco, Invitrogen Life Technologies Carlsbad, CA, USA) and visualized by electrophoresis in 1.5% (w/v) agarose gels and ethidium bromide staining. The inserts were then sequenced using Sp6 and T7 or M13 forward primers (DNA Core Facility, University of Alberta) and sequences were analyzed using BLAST software (NCBI, Bethesda, Maryland, USA). The testicular GH cDNA was compared with a consensus chicken pituitary GH cDNA based on Zhvirblis *et al.* (1987) (Gene Bank Accession # 211808), Lamb *et al.* (1988) (Gene Bank Accession # 63406), Baum *et al.* (1990) (Gene Bank Accession # 62909), Tanaka *et al.* (1992) (Gene Bank Accession # 222822), and Ip *et al.* (2001) (Gene Bank Accession # 9858171). This consensus sequence was identical to that reported by Tanaka *et al.* (1992).

Southern Blotting

cRNA Probe Synthesis: The 689 bp GH cDNA resulting from RT-PCR amplification of template cDNA with the oligonucleotide primers CLR3 and CLR5 was ligated into a PCR®II-TOPO® vector (Invitrogen Life Technologies Carlsbad, CA, USA) and cloned as detailed above. The plasmid containing the PCR product generated from

the GH specific primers was linearized with *BamH1* (Gibco, Invitrogen Life Technologies Carlsbad, CA, USA) and then purified by phenol/chloroform extraction and ethanol precipitation. The pellet was resuspended in 10 mM Tris-HCl, pH 8.0. The purified, linearized plasmid DNA was then digoxigenin (DIG) labeled by *in vitro* transcription of the DNA with DIG RNA labeling mix with the reagents and protocol from Roche Applied Science (Basel, Switzerland). Probe yield was estimated using DIG Quantification Teststrips (Roche Applied Science, Basel, Switzerland).

Blotting: GH cDNA amplified by PCR was transferred to a nylon membrane using the S&S Turbo Blotter and Blotting Stack Assembly for Alkaline Transfers (Schleicher & Schuell, Keene, NH, USA). After transfer, the DNA was fixed to the membrane by baking at 80 °C for 30 min in a vacuum oven. The blot was then prehybridized in 10 ml of DIG Easy Hyb hybridization buffer (Roche Applied Science, Basel, Switzerland) for 1 h at 50 °C. The DNA blot was then hybridized with 10 pmol DIG labeled RNA probe/ml hybridization buffer overnight at 50 °C. The hybridization buffer was poured off and the blot was washed 2 x 5 min in 2 x SSC (Roche Applied Science, Basel, Switzerland) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature, 2 x 5 min in 0.5 x SSC containing 0.1% SDS at 65 °C and 1 x 1 min in 0.3% (v/v) Tween® 20 in maleic acid buffer (10 mM maleic acid, 15 mM NaCl; pH 7.5) at room temperature. The blot was incubated in 1 x Blocking Reagent (Roche Applied Science, Basel, Switzerland) for 1 h with gentle shaking and then with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science, Basel, Switzerland)

diluted 1:5000 in 1 x Blocking Reagent. Colormetric detection of the DIG labeled probe was carried out using NBT/BCIP (Roche Applied Science, Basel, Switzerland).

Northern Blotting

Total cellular RNA was extracted from the pooled testes of three adult birds and subjected to Northern blot analysis using a DIG-labeled 689 bp GH cDNA, as detailed previously (Render *et al.*, 1995a). Briefly, the RNA was electrophoresed in 1% (w/v) agarose and 3.1% (w/v) formaldehyde gels and transferred to nylon membranes by capillarity. After transfer the RNA was immobilized in 60% (w/v) formamide (containing 0.75 mol NaCI/P, mmol pipes/P and 25 mmol EDTA/P, 0.2% (w/v) SDS, 1 X Denhart's reagent (0.1% w/v) ficoll, 0.1% (w/v) BSA, 0.1% (w/v) polyvinylpyrrolidine and 100 µg salmon sperm DNA/P, pH 6.8) in the presence of the probe (10 pmol/ml hybridization buffer) for 12 h at 55 °C. Following a brief rinse in 2 x SSC, the nylon membranes were washed at room temperature in 0.1% (w/v) SDS containing 2 x SSC and subsequently at 75 °C in 1% (w/v) SDS containing 0.1 x SSC. The blot was then incubated in Blocking Reagent (Roche Applied Science) for 1 h with gentle shaking and then with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science), diluted 1:5000 in 1 x Blocking Reagent. Colormetric detection of the DIG-labeled probe was carried out using BNT/BCIP (Roche Applied Science). Total cellular RNA from pooled adult pituitary glands was similar analyzed, for comparison.

III. RESULTS

GH mRNA: RT-PCR and Southern Blotting

As expected, RT-PCR of pituitary mRNA with oligonucleotide primers CLR3 and CLR5 generated a 689 bp cDNA fragment, which was not amplified with liver mRNA (Fig. 5.1A). A 689 bp fragment was, however, produced following RT-PCR of mRNA from the testis and the upper and middle vas deferens. This moiety was not produced with mRNA that had not been reverse-transcribed. The cDNA moieties generated from pituitary, testicular and vas deferens mRNA also hybridized with a DIG-labeled 689 bp riboprobe probe for GH mRNA, following southern blotting (Fig. 5.1B).

cDNA Sequencing

Sequencing of the 689 bp testicular GH cDNA showed it had >99.5% homology with the consensus published sequence for chicken pituitary GH cDNA. The sequence included 36 bps of the 5' untranslated region (UTR), the coding region (648 bp) for the 25 amino acid signal peptide and the 191 amino acid apoprotein, as well as 6 bps of the 3' UTR. This sequence only differed from the consensus pituitary GH cDNA by three base-pair substitutions (Fig. 5.2): a base substitution at residue 102 (137 of the cDNA fragment) (a T for C substitution), a base substitution at position 460 (496 of the cDNA fragment) (a G for A substitution) and a base substitution at position 587 (623 of the cDNA fragment) (an A for G substitution). The substitutions at residues 102 and 587 would not, however, change the amino acid sequence of the predicted protein, which would only differ from pituitary GH by a single amino acid (glycine for aspartate), at

position 154 of the 216 amino acid prohormone (amino acid position 129 of the 191 amino acid hormone) (99.5% homology of the predicted amino acid sequences).

Northern Blotting

As expected (Render *et al.*, 1995a), the 689 bp DIG-labeled GH riboprobe hybridized strongly to a 0.8 kb mRNA in the chicken pituitary gland (Fig. 5.3). This probe did not, however, hybridize to total mRNA extracted from the testis (Fig. 5.3).

Pit-1 mRNA: RT-PCR

RT-PCR of mRNA from the testis and upper vas deferens with oligonucleotides t14 and t15 for Pit-1 cDNA generated a 975 bp moiety identical to that in the pituitary gland (Fig. 5.4A). This moiety was not, however, generated with reverse transcribed mRNA from the liver (Fig. 5.4A), although a 500 bp cDNA moiety was generated from this in the presence of Gap DH primers, as with RNA from the other tissues (Fig. 5.4B).

GHR mRNA: RT-PCR

RT-PCR of mRNA from the testis with oligonucleotide primers for the extracellular (khu9 and khu10) and intracellular domains (khu11 and khu12) of the chicken GHR cDNA and for the full-length receptor (khu9 and khu12) amplified cDNA moieties identical in size (500 bp, 800 bp and 1350 bp respectively) to those amplified from hepatic mRNA (positive control) (Fig. 5.5). RT-PCR of reverse transcribed mRNA from the upper vas deferens with the same primer sets similarly generated 500 bp (khu9/khu10) and 800 bp (khu11/khu12) fragments, as also amplified with hepatic

mRNA (positive control) (Fig. 5.6A, B). These moieties were not generated with mRNA that had not been reverse-transcribed (data not shown). Endonuclease digestion with *Hae III* cleaved the 800 bp GHR cDNA moieties generated from liver and vas deferens mRNA into fragments of 470 bp and 330 bp (Fig. 5.6C).

IV. DISCUSSION

These results clearly demonstrate the presence of GH and GHR mRNA in the male reproductive tract and suggest autocrine/paracrine roles for GH in avian reproduction.

Although GH transcripts are present in the human testis, this is the first demonstration of GH mRNA in the testis of another species. Moreover, while GH gene expression occurs in the human testis, it is primarily the expression of the placental GH variant (hGH-V) rather than the pituitary GH gene (hGH-N) (Untergasser *et al.*, 1997, 1998; Berger *et al.*, 1999). The GH cDNA in the chicken testis, in contrast, closely resembles that in the pituitary gland.

The nucleotide sequence of chicken pituitary GH cDNA has been reported by several groups (Souza *et al.*, 1984; Zhvirblis *et al.*, 1987; Lamb *et al.*, 1988; Baum *et al.*, 1990; Tanaka *et al.*, 1992). Although all the published sequences have close homology (mostly >98%), a number of base-pair differences exist (Fig. 5.2) which may be species or strain related (Baum *et al.*, 1990), as numerous intronic DNA polymorphisms have also been identified in different chicken strains (Kuhnlein *et al.*, 1997; Feng *et al.*, 1997; Ip *et al.*, 2001). The three base-pair substitutions in testicular GH cDNA are not, however, species or strain related, since they are unique and, in the same birds, are not

present in pituitary GH cDNA, brain GH cDNA, bursal GH cDNA, thymic GH cDNA, or splenic GH cDNA (Render *et al.*, 1995a,b). It is therefore likely that GH gene transcription is tissue-specific in the domestic fowl. The recent discovery of a novel GH cDNA in the chicken retina (Takeuchi *et al.*, 2001) supports this possibility, as does the differential transcription of the GH gene family in the human testis (Untegasser *et al.*, 1997; Berger *et al.*, 1999). The cDNA of testicular GH also differs from the retinal GH cDNA, since the base-pair substitutions in testicular GH (at positions 460 and 587) are not present in retinal GH cDNA (Takeuchi *et al.*, 2001).

The predicted amino acid sequence coded by chicken testicular GH cDNA only differs from pituitary GH by a single residue (glycine for aspartate at position 154 of the 216 amino acid prohormone, position 129 of the mature hormone). The 191 amino acid apoprotein has 4 conserved cysteine residues (at positions 53, 164, 181 and 189) that are thought to be responsible for the formation of 2 intramolecular or intermolecular disulfide bridges and the structural folding of the molecule for receptor recognition. It also has the conserved domains between amino acids 82-87, 115-120, and 153-162 that are also thought to be important for receptor binding or for specific biological functions (Foster *et al.*, 1990). It is therefore highly likely that the coded protein would have biological activity.

The GH message in the chicken testis is of low abundance, since it could not be detected by northern blotting, although this technique readily detects pituitary GH mRNA. The testicular GH transcript is, however, likely to be translated, since proteins in the chicken testis with GH-immunoreactivity have been reported in preliminary studies (Harvey *et al.*, 2002), although GH has yet to be determined in the testis of other species.

Immunoreactive GH has, however, been located in the Wolffian ducts of fetal mice (Nguyen *et al.*, 1996) and the Wolffian ducts of embryonic chicks (Wang, 1989; Harvey *et al.*, 2000a). It is therefore of interest that GH mRNA was also detected in the vas deferens and the cloaca, indicating that they too are sites of GH synthesis. Moreover, as GHR transcripts are present throughout the male reproductive tract, GH may have local autocrine/paracrine actions in these tissues. The presence of GH in the reproductive tracts of embryonic chicks prior to the differentiation of the pituitary gland and the appearance of GH in plasma (Harvey *et al.*, 2000a) strongly supports this possibility.

It is now well established that the testis and the male reproductive tract are target sites for GH action (Hull and Harvey, 2000a, 2000b). Indeed, GH is thought to be essential for testicular growth and development, since GH deficiency is associated with abnormally small testes (Spiteri-Grech and Nieschlag, 1992). The differentiation of the Wolffian duct is similarly thought to be GH-dependent, since GH immunoneutralization blocks Wolffian duct differentiation in fetal mice, whereas GH administration restores normal development of the reproductive tract (Nguyen *et al.*, 1996). Numerous actions of GH in gametogenesis (Gravance, 1997; Ovesen *et al.*, 1998) and steroidogenesis (Shohan *et al.*, 1992; Kanzaki and Morris, 1999) have also been documented and GH deficiency is thought to be causally linked to male infertility (Shohan *et al.*, 1992; Shimonovitz *et al.*, 1993).

These actions of GH are likely to be receptor mediated, especially as GHR immunoreactivity and GHR mRNA are abundantly present in the Wolffian ducts, epididymis, vas deferens, seminal vesicles, prostate and testis (Leydig cells, Sertoli cells, spermatogonia, spermatocytes) of fetal and adult rats (Lobie *et al.*, 1990; Garcia-Aragon

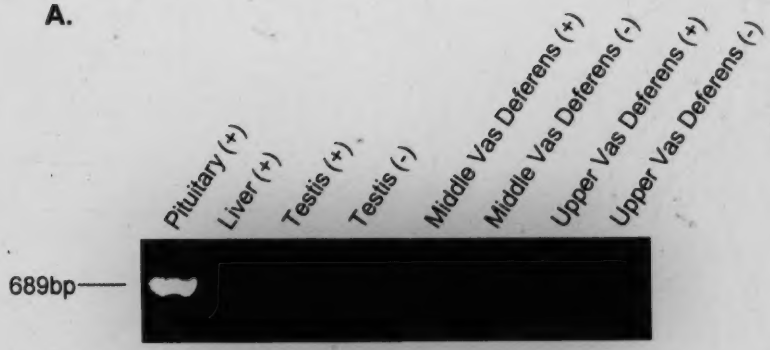
et al., 1992; Reiter *et al.*, 1999). The demonstration of GHR mRNA in the testis, and vas deferens of male chickens, for the first time, similarly suggests testicular and extra - testicular actions of GH in avian reproductive function. As some of these are sites of GH production and GH action, it is possible that GH has local autocrine/paracrine roles in the male reproductive tract, as in many other peripheral tissues (Harvey and Hull, 1997; Harvey *et al.*, 1998).

The expression of the GH gene in the pituitary is thought to be dependent upon the Pit-1 transcription factor (Harvey *et al.*, 2000b). It is therefore of interest that Pit-1 mRNA was also detected in the chicken testes and vas deferens. This may, therefore, indicate a role for Pit-1 in testicular GH expression. Pit-1 is, however, also present in placental tissue but it has no role in the expression of the placental GH variant. Indeed, the factors regulating GH expression in extrapituitary sites are largely unknown (Harvey and Hull, 1997) and the presence of GH-releasing hormone (Matsubara *et al.*, 1995; Olchovsky *et al.*, 1996), thyrotropin-releasing hormone (Wilber and Xu, 1998; Li *et al.*, 2002), ghrelin (Tanaka *et al.*, 2001; Tena-Sempere *et al.*, 2002), somatostatin (Pekary *et al.*, 1984; Zhu *et al.*, 1998) and insulin-like growth factors (Morera *et al.*, 1987; Baker *et al.*, 1996) in testicular tissue may also be unrelated to GH regulation (Campbell and Scanes, 1995), in contrast with the roles of these factors in pituitary GH secretion.

In summary, the results of these studies demonstrate the presence of GH-, GHR- and Pit-1 mRNA in the male reproductive system, in which GH may have local autocrine/paracrine roles in reproductive function.

Fig 5.1 RT-PCR and Southern Blotting for GH in male chicken reproductive tissues. (A) RT-PCR of growth hormone (GH) mRNA in the testis and reproductive tract of male chickens. Total tissue RNA was extracted and amplified in the presence of oligonucleotide primers (CLR3 and CLR5) designed to amplify a 689 bp fragment of pituitary GH cDNA (Render *et al.*, 1995a). This fragment was amplified with reverse transcribed RNA (+) extracted from the testis, lower vas deferens, middle vas deferens and cloaca and stained by ethidium bromide. This fragment was not evident when PCR was performed with RNA in the absence of reverse transcriptase (-). The presence and absence of this fragment in amplified pituitary and liver cDNA is shown for comparison. (B) Southern blotting of PCR products amplified in the presence of oligonucleotide primers designed to amplify a 689 bp fragment of pituitary GH cDNA, using a DIG-labeled 689 bp pituitary GH riboprobe. RNA from the testis, and the upper and middle vas deferens produced a 689 bp GH cDNA after reverse transcription (+), but not in the absence of reverse transcriptase (-).

A.


Pituitary (+)
Liver (+)
Testis (+)
Testis (-)
Middle Vas Deferens (+)
Middle Vas Deferens (-)
Upper Vas Deferens (+)
Upper Vas Deferens (-)
689bp

B.

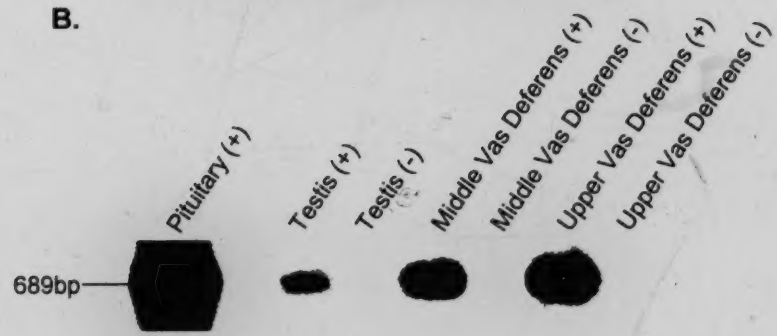
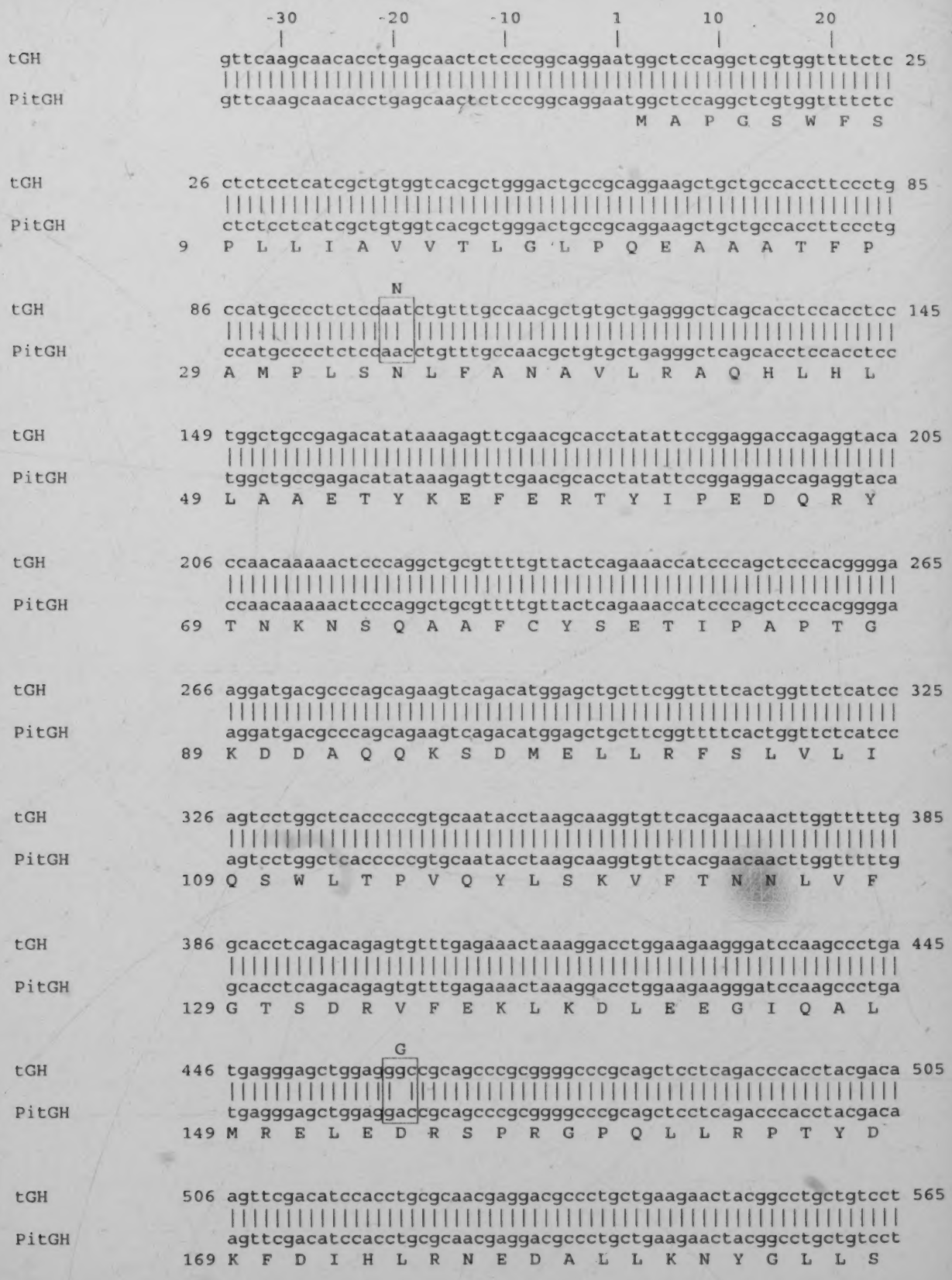

Pituitary (+)
Testis (+)
Testis (-)
Middle Vas Deferens (+)
Middle Vas Deferens (-)
Upper Vas Deferens (+)
Upper Vas Deferens (-)
689bp

Fig 5.2 Comparison of testicular cGH nucleotide sequence with pituitary cGH. Nucleotide and amino acid sequence of testicular growth hormone (GH) cDNA (tGH), in comparison with pituitary GH cDNA (Pit-GH) (Tanaka *et al.*, 1992). The initiation site is the ATG codon for methionine. The 5' flanking untranslated region is indicated as -36 to -1. The first 25 amino acids are the signal sequence for the 216 amino acid prohormone. Base-pair substitutions are indicated in the boxed codons. The base-pair substitution at position 460 of the coding region would result in a GGC codon and code for glycine rather than aspartate. In comparison with the sequence reported by Tanaka *et al.*, (1992), the pituitary GH cDNA reported by Lamb *et al.*, (1988) differs by base-pair substitutions at positions153 (T for C), 162 (C for T) and 302 (C for T). The pituitary GH cDNA reported by Baum *et al.*, (1990) differs by base-pair substitutions at positions 336 (G for C), 465 (A for C), 466 (T for A), 467 (C for G), 468 (A for C) and 504 (T for C). The pituitary GH cDNA reported by Zhvirblis *et al.*, (1987) differs by base-pair substitutions at position 336 (G for C) and 339 (T for C). None of these substitutions were present in testicular GH cDNA.


-30 -20 -10 1 10 20
tGH gttcaagcaacacctgagcaactctcccggcaggaatggctccaggctcgtggttttctc 25
PitGH gttcaagcaacacctgagcaactctcccggcaggaatggctccaggctcgtggttttctc
M A P G S W F S
tGH 26 ctctcctcatcgctgtggtcacgctgggactgccgcaggaagctgctgccaccttccctg 85
PitGH ctctcctcatcgctgtggtcacgctgggactgccgcaggaagctgctgccaccttccctg
9 P L L I A V V T L G L P Q E A A A T F P
N
tGH 86 ccatgcccctctccaatctgtttgccaacgctgtgctgagggctcagcacctccacctcc 145
PitGH ccatgcccctctccaacctgtttgccaacgctgtgctgagggctcagcacctccacctcc
29 A M P L S N L F A N A V L R A Q H L H L
tGH 149 tggctgccgagacatataaagagttcgaacgcacctatattccggaggaccagaggtaca 205
PitGH tggctgccgagacatataaagagttcgaacgcacctatattccggaggaccagaggtaca
49 L A A E T Y K E F E R T Y I P E D Q R Y
tGH 206 ccaacaaaaactcccaggctgcgttttgttactcagaaaccatcccagctcccacgggga 265
PitGH ccaacaaaaactcccaggctgcgttttgttactcagaaaccatcccagctcccacgggga
69 T N K N S Q A A F C Y S E T I P A P T G
tGH 266 aggatgacgcccagcagaagtcagacatggagctgcttcggttttcactggttctcatcc 325
PitGH aggatgacgcccagcagaagtcagacatggagctgcttcggttttcactggttctcatcc
89 K D D A Q Q K S D M E L L R F S L V L I
tGH 326 agtcctggctcacccccgtgcaatacctaagcaaggtgttcacgaacaacttggtttttg 385
PitGH agtcctggctcacccccgtgcaatacctaagcaaggtgttcacgaacaacttggtttttg
109 Q S W L T P V Q Y L S K V F T N N L V F
tGH 386 gcacctcagacagagtgtttgagaaactaaaggacctggaagaagggatccaagccctga 445
PitGH gcacctcagacagagtgtttgagaaactaaaggacctggaagaagggatccaagccctga
129 G T S D R V F E K L K D L E E G I Q A L
G
tGH 446 tgagggagctggagggccgcagcccgcggggcccgcagctcctcagacccacctacgaca 505
PitGH tgagggagctggaggaccgcagcccgcggggcccgcagctcctcagacccacctacgaca
149 M R E L E D R S P R G P Q L L R P T Y D
tGH 506 agttcgacatccacctgcgcaacgaggacgccctgctgaagaactacggcctgctgtcct 565
PitGH agttcgacatccacctgcgcaacgaggacgccctgctgaagaactacggcctgctgtcct
169 K F D I H L R N E D A L L K N Y G L L S

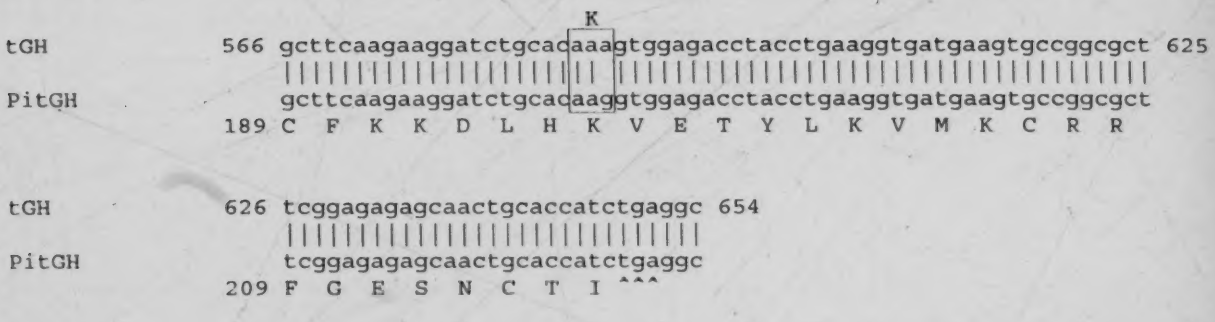

K
tGH 566 gcttcaagaaggatctgcacaaagtggagacctacctgaaggtgatgaagtgccggcgct 625
PitGH gcttcaagaaggatctgcacaaggtggagacctacctgaaggtgatgaagtgccggcgct
189 C F K K D L H K V E T Y L K V M K C R R
tGH 626 tcggagagagcaactgcaccatctgaggc 654
PitGH tcggagagagcaactgcaccatctgaggc
209 F G E S N C T I ^^^

Fig 5.3 Northern blotting for growth hormone (GH) mRNA in the pituitary and testis of adult chickens. Hybridization of a 689 bp DIG-labeled GH riboprobe is shown for pituitary RNA, but not for testicular RNA.

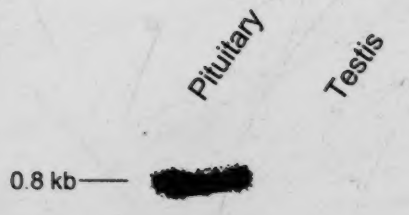

Pituitary
Testis
0.8 kb

Fig 5.4 RT-PCR for Pit-1 and Gap DH in male chicken reproductive tissues. (A) Pit-1 mRNA in the testis and vas deferens of chickens. Total tissue RNA was extracted and amplified by RT-PCR in the presence of oligonucleotide primers t14 and t15, designed to generate a 975 bp fragment of chicken Pit-1 cDNA (Harvey *et al.*, 2000). The generation of this cDNA with pituitary RNA and its absence following RT-PCR with liver RNA acted as positive and negative controls, respectively (Harvey *et al.*, 2000).
(B) The specificity of the Pit-1 PCR is indicated by the generation of 500 bp Gap DH cDNA fragments following PCR of reverse transcribed (+) RNA from the testis, vas deferens and liver. No signal was observed in the absence of reverse transcriptase (-).

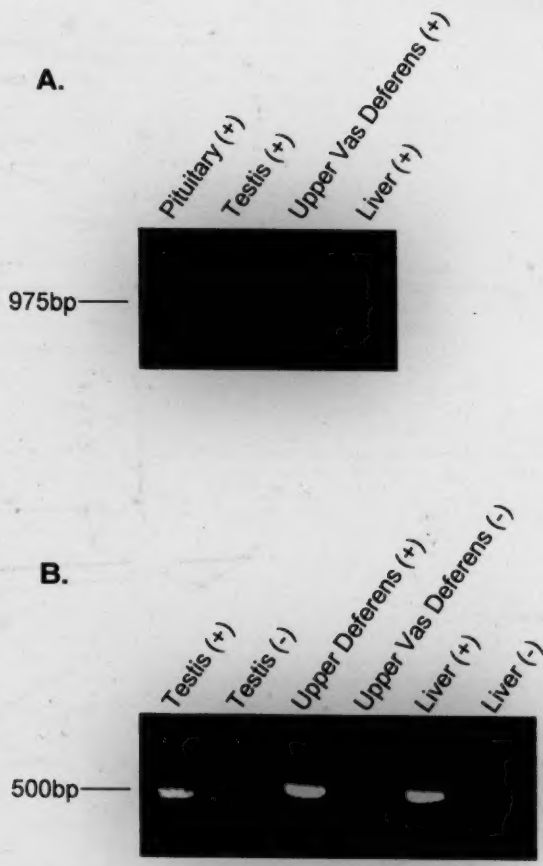

A.
Pituitary (+)
Testis (+)
Upper Vas Deferens (+)
Liver (+)
975bp
B.
Testis (+)
Testis (-)
Upper Deferens (+)
Upper Vas Deferens (-)
Liver (+)
Liver (-)
500bp

Fig 5.5 Growth hormone receptor (GHR) mRNA in the testis of male chickens. Total tissue RNA from the testis was amplified by RT-PCR in the presence of oligonucleotide primers khu9 and khu10, khu11 and khu12 or khu9 and khu12, designed to generate 500 bp, 800 bp and 1350 bp fragments, respectively, of the chick GHR cDNA (Hull *et al.*, 1995). The generation of similar cDNA fragments from reverse transcribed liver RNA is shown as a positive control.

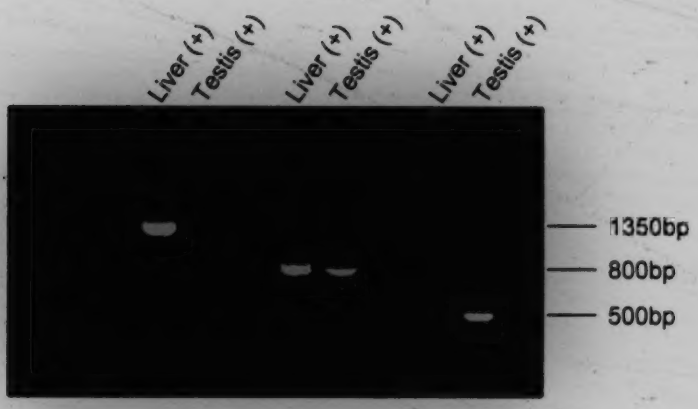
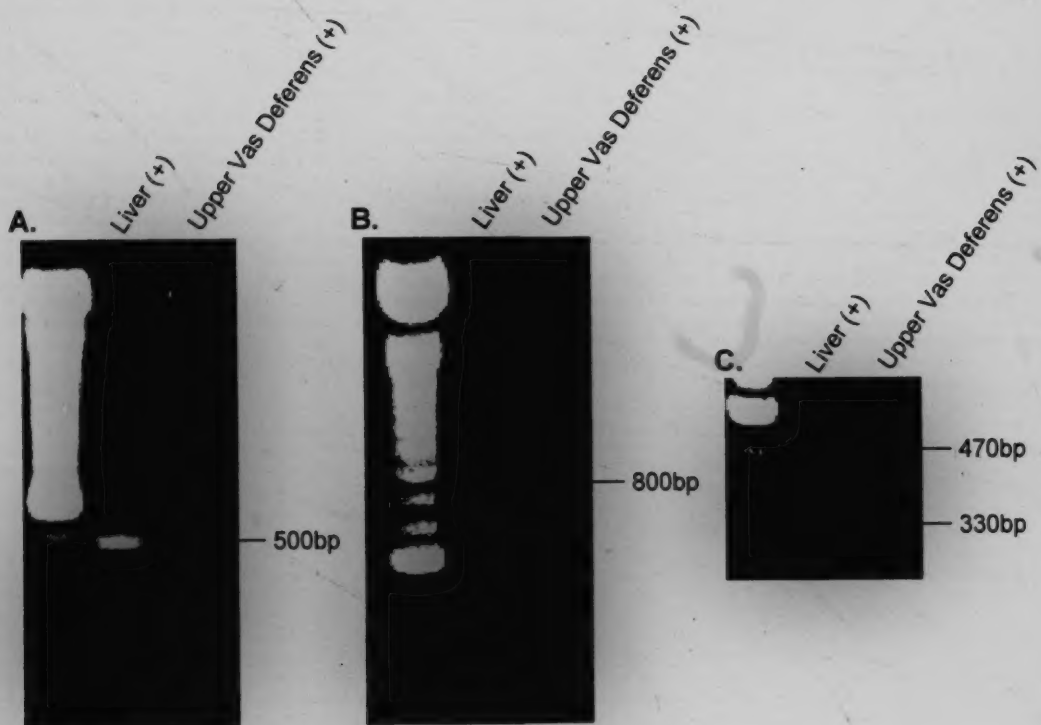

Liver (+)
Testis (+)
Liver (+)
Testis (+)
Liver (+)
Testis (+)
1350bp
800bp
500bp

Fig 5.6 Growth hormone receptor (GHR) mRNA in the reproductive tract of male chickens. Total tissue RNA from the upper vas deferens was amplified by RT-PCR in the presence of oligonucleotide primers khu9 and khu10 or khu11 and khu12 (Hull *et al.*, 1995), designed to generate a 500 bp fragment of the GHR cDNA coding for the extracellular domain of the GHR (A) or with primers khu11 and khu12, designed to generate a 800 bp fragment of the GHR cDNA coding for the intracellular domain of the GHR (Hull *et al.*, 1995) (B). The generation of these fragments with hepatic cDNA provided a positive control. (C) Endonuclease digestion of growth hormone receptor (GHR) cDNA. The GHR cDNAs generated from the RT-PCR of hepatic and vas deferens mRNA (with oligonucleotide primers for the intracellular domains of the chicken GHR cDNA) were digested with *Hae III*. Fragments of appropriate size (470 bp and 330 bp) indicated sequence similarly with the full length chicken GHR cDNA (Hull *et al.*, 1995).


A.
Liver (+)
Upper Vas Deferens (+)
500bp
B.
Liver (+)
Upper Vas Deferens (+)
800bp
C.
Liver (+)
Upper Vas Deferens (+)
470bp
330bp

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Chapter 6

General Discussion

I. OVERVIEW

The results of these studies clearly demonstrate the presence of pituitary hormones (GH and TSH) in extrapituitary tissues of the chick embryo, prior to the differentiation of the pituitary gland.

These results confirm and extend similar results for GH that were recently published by Harvey *et al.* (1998, 2000, 2001a). These results more clearly identify the location of GH immunoreactivity within central and peripheral tissues and demonstrate, using two fixatives, that this is not an immunohistochemical artifact. The recent finding of GH expression in the heart of ED 7 chick embryos (Takeuchi *et al.*, 2001) and the headless bodies of ED 2-ED 7 embryos (Harvey *et al.*, 2001b) strongly suggests the presence of GH in early chick embryos arises from the local expression of a GH gene.

Although the presence of GH in extrapituitary tissues of the chick embryo was expected, it served as a positive control for immunohistochemical studies on TSH localization. This is the first study to demonstrate TSH immunoreactivity in central and peripheral extrapituitary tissues during embryogenesis, although it confirms previous reports of TSH in the brain (Hojvat *et al.*, 1982a,b) and gut (Wang *et al.*, 1997) of fetal and adult rats. Interestingly, the results of these studies clearly show tissue-specific differences in the distribution of TSH and GH in the chick embryo, possibly reflecting differences in gene regulation, hormone action or tissue function. The distribution of GH and TSH also reflects the distribution of Pit-1 in some tissues but not in others. The extrapituitary expression of the GH and TSH genes is therefore unlikely to be Pit-1 dependent, and Pit-1 is clearly not a pituitary-specific transcription factor in early embryos. Indeed, prolactin immunoreactivity is also widely present in ED 7 embryos

(unpublished observation) and this too is likely to be Pit-1 independent, in contrast to the expression of GH, TSH and prolactin in the mammalian pituitary gland (Burrows *et al.*, 2001). The finding of TSH in extrapituitary tissues during embryogenesis is also consistent with the localization of a pituitary glycoprotein, LH, in chick embryos (Shirasawa *et al.*, 1996), although LH-immunoreactivity was of much less abundance and restricted to specific cells in the lung and alimentary tract.

The extrapituitary presence of GH in some tissues (e.g. the heart, liver and mesonephros, Harvey *et al.*, 1998; e.g. the eye, Takeuchi *et al.*, 2001) is thought to diminish during embryogenesis, although it persists in the brain (Render *et al.*, 1995a) and immune tissues (Render *et al.*, 1995b) of adult chickens. The results of these studies also demonstrate that GH expression persists in the gonad and reproductive tract of male domestic fowl. Furthermore, these results demonstrate the presence of a testis-specific variant of the GH gene, whereas the GH gene in the chicken brain, thymus, liver and spleen is identical to the pituitary GH gene (Render *et al.*, 1995a,b). This finding is, however, consistent with the presence of a novel GH gene variant in the eyes of embryonic chicks (Takeuchi *et al.*, 2001), although this variant was not detected in the male reproductive tract (unpublished observations).

II. THE EXPANDING ENDOCRINE SYSTEM

The presence of pituitary hormones in extrapituitary tissues of the chick is consistent with numerous studies that have now demonstrated this phenomenon throughout the vertebrates (Roth *et al.*, 1982; Kreiger, 1983; Smith *et al.*, 1983; Mandrekar *et al.*, 1990; Harvey *et al.*, 1993). Moreover, although *ectopic hormone*

syndromes were once thought to be a manifestation of malignancy (Morgello *et al.*, 1988; Coleman *et al.*, 2000; Indinnimeo *et al.*, 2001) many peripheral hormones are now known to be widely expressed physiologically (Roth *et al.*, 1982; Harvey *et al.*, 1993; Hull *et al.*, 1998; Wu *et al.*, 1999; Huang *et al.*, 2001). Furthermore, every cell in an individual contains the same genome and, therefore, it is not surprising that most tissues have "endocrine" function, particularly during early development. Indeed, many endocrine systems appear to be developmentally regulated and this may provide a mechanism to limit or restrict hormone production to specific sites. The presence of GH, for instance, in fetal tissues is far greater than in the tissues of adults (Wu *et al.*, 1996; Recher *et al.*, 2000).

The presence of GH and TSH in the chick embryo occurs prior to the ontogenic differentiation of the pituitary gland. GH and GH mRNA have similarly been found at ED 2 and ED 3 (Harvey *et al.*, 2000; 2001a), prior to the differentiation of the cardiovascular system. The presence of GH in early embryos is therefore unlikely to have endocrine roles during development. Indeed, GH and GH mRNA have recently been found in zygotes from the two-cell stage and in the morulla and blastocyst (Pantaleon *et al.*, 1997; Yang *et al.*, 1999; Kolle *et al.*, 2001), preceding the development of endocrine communication. Moreover, although GH is produced in many extrapituitary tissues (reviewed by Harvey and Hull, 1997), extrapituitary GH is unlikely to significantly contribute to GH in the systemic circulation, since plasma GH levels are undetectable following pituitary ablation or hypophysectomy (Harvey and Hull, 1997). It is therefore likely that GH (and TSH) in extrapituitary tissues acts locally in autocrine or paracrine ways. The widespread presence of GH-receptors and GH-responsive genes in

tissues of early chick embryos (Harvey *et al.*, 1998, 2000, 2001a,b) supports this view, as does the demonstration of endogenous GH actions in preimplantation murine and bovine embryos that are blocked by GH immunoneutralization (Pantaleon *et al.*, 1997; Izadyar *et al.*, 2000). These actions (e.g. the stimulation of glucose uptake and hatching of preimplantation blastocysts; Pantaleon *et al.*, 1997; Kolle *et al.*, 2001), may be tissue and temporally specific and unrelated to the actions of endogenous GH in later development. Harvey and Hull (1997), in contrast, have suggested that the actions of GH locally may reflect an "emergency" mechanism that accelerates cellular pathways that are "strategically" regulated by pituitary GH in an endocrine way. It is, therefore, pertinent that GH and other "pituitary" hormones have been detected in invertebrates lacking pituitary glands (Tsushima *et al.*, 1974; Roth *et al.*, 1982; Verhaert *et al.*, 1986; Kawauchi *et al.*, 1990) and hence lacking the "endocrine" roles that these hormones have in vertebrates. It is thus likely that GH (and TSH) evolved phylogenetically as local messenger molecules, with roles dissimilar from those associated with pituitary gland function in vertebrates. The naming of a messenger molecule with respect to a biological function that it induces is therefore inappropriate, especially as it encourages dogmas that often hinder scientific enquiry (Kastin *et al.*, 1984).

III. LIMITATIONS OF THE STUDY

The methodologies of the present study have a number of limitations. Immunohistochemistry is a widely used and accepted technique. However, as Chapter 4 clearly indicates, the method of fixation can drastically affect the results in the same tissue with the same antibody and identical processing. In this study Carnoy's solution

(an alcohol based, precipitating fixative) was shown to mask the GH antigen in ED 7 chick embryo tissues and drastically reduce the amount of visible specific staining compared to 4% paraformaldehyde fixed sections. Therefore, it is difficult to conclude that a peptide is not present when using immunohistochemistry because the method of fixation may hide the antigen in question. Therefore, the presence of βTSH and Pit-1 should be re-evaluated in 4% paraformaldehyde fixed sections to ensure their presence was not underestimated. Antibodies can, alternatively, bind non-specifically to other antigens in tissues, leading to an artefactual overestimation of staining. However, this is likely not the case in this study. The high titre of the GH antibody and the high dilution (1:1000) used suggests it is unlikely to bind non-specifically to other proteins. In addition, Harvey and Scanes (1977) determined by cross reactivity studies that the antibody used for GH detection was specific for GH, and did not cross-react with other pituitary hormones, even at concentrations of 1 mg/ml. The Pit-1 antibody used in this study could, however, have been more specific if it was against chicken Pit-1 instead of rat Pit-1. However, this antibody has been used successfully in other species and the results of the Western analysis indicate it was reacting with a peptide of the appropriate size (33 kDa), and was therefore probably specific for chicken Pit-1. The anti-βTSH antibody was also directed against rat βTSH. The βTSH staining would be more reliable if an antibody directed against chicken βTSH had been used, or if Western analysis had been performed to ensure the antibody was detecting the correct size of protein. However, the anti-TSH antibody does not cross react with LH and FSH, so it is thought to be specific for TSH.

Although RT-PCR is a very sensitive method , rare transcripts may not be sufficiently abundant in some tissue extracts to be visibly seen on an agarose gels following RT-PCR amplification.. This methodological limitation probably accounts for the apparent absence of the Pit-1 transcript in embryo tissues subjected to RT-PCR and ethidium bromide staining, since the transcript was detectable following Southern blotting. As Southern blotting is far more sensitive than ethidium bromide staining, this procedure should, therefore be routinely used in conjunction with RT-PCR for the screening of transcript distribution. Comprehensive controls (tissue positive and negative controls) should also be routinely used with this technique, to ensure there is no genomic DNA contamination of the extracts and that the amplified cDNA fragments are the same size as those in the positive controls. Sequencing of the PCR products should also be undertaken to ensure the primers actually amplify the gene of interest.

GH, TSH and Pit-1 may be transiently expressed in the chick embryo at different times of development. Therefore, different stages of chick embryos, throughout embryonic development, should be used for immunohistochemical studies to determine if these peptides are present throughout development and whether the tissue distribution of the proteins changes with embryonic age.

IV. FUTURE STUDIES

This study clearly demonstrates the presence of GH, βTSH and Pit-1 in the ED 7 chick embryo, and suggests that the extrapituitary distribution of pituitary hormones is a common occurrence. Indeed, Shirasawa *et al.* (1996) identified LH in the developing lung of chick embryos and it would, therefore, be of interest to perform

immunohistochemistry (IHC) for the anterior pituitary hormones not fully studied in this thesis (LH, FSH, PRL and ACTH) to determine their distribution in the ED 7 chick embryo. This method localizes the protein in the tissue and, therefore, *in situ* hybridization could be performed in the same sections to localize the gene transcript for these hormones. IHC and *in situ* hybridization could be done on the same sections at the same time to co-localize the gene transcript and the protein, which could indicate whether the protein is actually synthesized in the tissue of localization, or simply sequestered from the blood.

This study has demonstrated the presence of GH and TSH in embryonic chick tissues, but provides no evidence that these hormones are secreted from these tissues. The reverse hemolytic plaque assay could be used to determine which tissues secrete the hormone since it can detect hormone secretion from individual cells (Neill and Frawley, 1983), and has previously been used by Porter (1997) to detect GH secretion from embryonic chick pituitary glands. Radioimmunoassays or ELISAs could also be used to investigate hormone secretion from whole tissues using different stimulatory or inhibitory factors to investigated secretory control. If GH and βTSH were secreted from cultured embryonic tissues in a regulated way, it would support the idea that these hormones have autocrine/paracrine roles in embryogenesis.

If these hormones are produced and secreted from extrapituitary tissues of the embryo, it would be of interest to determine what functions they have during embryogenesis and, similarly, what roles GH might have in the adult male reproductive tract. Functional studies would involve investigation into the hormones' mechanisms of action, and the intracellular sites of action. Sense oligonucleotides or

immunoneutralization could be used to block functional GH and TSH or their receptors and determine what occurs in the embryo or reproductive tract when these local hormones are unavailable. However, since many factors are likely to regulate the transcriptional activity of growth and differentiation, GH or TSH knockout may not impair early embryonic development. In fact, Snell dwarf mice, having a Pit-1 mutation and a lack of pituitary GH and TSH, have normal early embryonic development (Gage *et al.*, 1996), as do GH-receptor knockout mice (Bartke *et al.*, 1999). Another useful method for determining a particular hormone's action in a particular embryonic tissue would be the construction of a replication-defective retrovirus that carries an inserted gene of interest (Tickle, 1992). The viruses can be injected into specific embryonic tissues and will spread locally as development progresses. Dominant mutant receptor genes for GH and βTSH that result in non-functional receptors could be introduced to study the effects of GH and βTSH on individual embryonic tissues.

V. SUMMARY

In summary, this study has demonstrated the presence of GH-, βTSH- and Pit-1-like proteins in the ED 7 chick embryo by immunohistochemistry. Western analysis revealed that a Pit-1-like protein of identical molecular size to pituitary Pit-1 is present in some tissues of the ED7 chick embryo. RT-PCR and Southern blotting also reveal the presence of Pit-1 mRNA in embryo tissues the same size as in the pituitary. These results strongly indicate the presence of GH, βTSH and Pit-1 in the ED 7 embryo and raises questions concerning their sites of synthesis and potential actions. Also, RT-PCR has revealed the presence of GH, GHR and Pit-1 mRNA in the adult male chicken

reproductive tract and raises questions concerning GH synthesis and actions in chicken reproductive physiology. The identification of a novel GH transcript in the adult chicken testis indicates a possible novel role for testicular GH in the chicken. Together these results suggest pituitary hormones are widely distributed in extrapituitary tissues of chick embryos and adults and may act locally as autocrines or paracrines.

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